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EFFECT OF HEAVY METALS FOUND IN FLUE GAS ON GROWTH AND LIPID
ACCUMULATION FOR GREEN ALGAE *SCENEDESMUS OBLIQUUS*

by

Reece Sansom Butler

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Mechanical Engineering

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Logan, Utah

2011

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ABSTRACT

Effect of Heavy Metals Found in Flue Gas on Growth and Lipid Accumulation for Green

Algae Scenedesmus obliquus

by

Reece Butler, Master of Science

Utah State University, 2011

Major Professor: Dr. Byard Wood
Department: Mechanical Engineering

This study evaluated the effect of several heavy metals that are present in flue gases on the algae, focusing on the growth and accumulation of lipids in the algae that can be converted to biodiesel. Concentrations for the heavy metals were calculated based on literature and assumptions. Metals were tested individually first at the highest concentrations that might be present (reference concentrations). The metals and their reference concentrations were: arsenic at 1.56 mg/L, cadmium at 0.3 mg/L, chromium at 2.6 mg/L, cobalt at 0.32 mg/L, copper at 2.62 mg/L, lead at 1.09 mg/L, nickel at 5.08 mg/L, mercury at 0.2 mg/L, selenium at 0.2 mg/L, and zinc at 8.8 mg/L. At these concentrations, most of the metals had a negative effect on the growth and lipid content of the algae. All of the metals were then tested at lower concentrations. At 1/20 the reference concentrations, the metals enhanced growth as well as lipid accumulation in the algae. At higher concentrations there was a negative effect.

(83 pages)

PUBLIC ABSTRACT

In recent years there has been a growing interest within the United States to replace fossil fuels with biofuels for environmental and national security reasons. Algal feedstocks have the potential of producing significantly higher yields of oil for biofuels than any other source. It is well known that high concentrations of CO₂ can increase algal yields. Flue gas from coal fire power plants is an obvious source of CO₂. The flue gas, however, will contain other chemicals besides CO₂ that may not be beneficial to the algae. The research objectives for this study were to determine how heavy metals present in the flue gas would affect the production of biodiesel, which is determined by the growth of the algae and the content of lipids inside the algae. This study found that the effects are concentration dependent, and that at concentrations likely to be present in flue gases, production of biofuels from algae should not be affected. This study did find, however, that at high concentrations the growth and lipid content of the algae will be negatively affected.

This work benefits the biofuels community in that there has been much interest in using flue gas CO₂ to enhance production of algae, but the effect that the heavy metals would have on that production had not been studied. This study shows that algae can be grown with flue gas CO₂ without having the production of biofuels be negatively affected by the metals (if concentrations remain below the acceptable levels).

This project was funded by Arizona Public Service Company (APS), The US Department of Energy, and Utah State University. The total expenditures of these projects was approximately \$170,000.

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Reece Butler

PREFACE

This thesis research was completed in two phases. The initial funding for this project was provided by Arizona Public Service (APS). Consequently, the initial phase of this project was influenced strongly by APS's needs and requirements of the sponsored project that included:

- Use of *Scenedesmus obliquus* as the algal strain for the study
- Testing protocol
- Media
- Reference concentrations of heavy metals in coal fired flue gases

Mid-way through the project, APS made a corporate decision to terminate its algal biomass research program. This decision by APS provided the opportunity to expand the objectives of the project using internal funds and subsequently sponsorship from US Department of Energy (DOE).

For this thesis project, the supervisory committee approved the continued use of *Scenedesmus obliquus* and media, a change in the testing protocol to include three separate tests for each variable, and inclusion of all the data gathered under APS sponsorship. Otherwise, it would have taken more than an additional six months to satisfy the research objectives.

CONTENTS

	Page
ABSTRACT	iii
PUBLIC ABSTRACT	iv
ACKNOWLEDGMENTS	v
PREFACE	vi
LIST OF TABLES	ix
LIST OF FIGURES	xi
INTRODUCTION	1
LITERATURE REVIEW	3
How CO ₂ levels affect the environment.....	3
Algae could utilize waste CO ₂ and produce biodiesel	4
Producing algae on a large scale to absorb flue gas CO ₂	9
Chemical species found in flue gas and their effect on algae	13
Effects that heavy metals in flue gas could have on the algae	14
EXPERIMENTAL SETUP AND PROCEDURES	17
Reactors used for experiments	17
Inoculation procedure.....	17
Media used	19
Determination of metals concentration	20
Sampling.....	21
Measurement of growth with OD and conversion from OD to TSS.....	22
Transesterification	23
Statistical analysis	23
Uncertainty analysis	24
RESULTS	28
Introduction of results	28
Zinc, lead, cobalt, and chromium	28
Mercury, nickel, copper, and cadmium	32
Selenium	35
Arsenic	37

Summary of individual metals	40
All metals at different concentrations	41
Summary of combined metals results	45
ADDITIONAL DISCUSSION	47
Concentration of metals	47
Using different reactors could change results	49
Using a different strain/media could change results	51
Presence of chelators could change results	54
Differences in pH could change results.....	55
CONCLUSIONS.....	56
REFERENCES	58
APPENDICES	63
APPENDIX A	64
APPENDIX B	65
APPENDIX C	67
APPENDIX D	68
APPENDIX E.....	70

LIST OF TABLES

Table		Page
1	Highest Mass Fractions [42] and Reference Metal Concentrations Calculated from Literature (Appendix B).....	21
2	Studentized range distribution used for Tukey's test [50].	25
3	Mean Values \pm 95% Confidence Region for Growth of <i>Scenedesmus obliquus</i> with Zinc, Lead, Cobalt, and Chromium at Their Given Concentrations Versus the Control with No Metals	30
4	Mean Values \pm 95% Confidence Region for Lipid Analysis of <i>Scenedesmus obliquus</i> with Zinc, Lead, Cobalt, and Chromium at Their Given Concentrations Versus the Control with No Metals	31
5	Mean Values \pm 95% Confidence Region for Growth of <i>Scenedesmus obliquus</i> with Mercury, Nickel, Copper, and Cadmium at Their Given Concentrations Versus the Control with No Metals	33
6	Mean Values \pm 95% Confidence Region for Lipid Analysis of <i>Scenedesmus obliquus</i> with Mercury, Nickel, Copper, and Cadmium at Their Given Concentrations Versus the Control with No Metals	35
7	Mean Values \pm 95% Confidence Region for Growth of <i>Scenedesmus obliquus</i> with Selenium at Its Given Concentration Versus the Control with No Metals.....	36
8	Mean Values \pm 95% Confidence Region for Lipid Analysis of <i>Scenedesmus obliquus</i> with Selenium at Its Given Concentration Versus the Control with No Metals.....	37
9	Mean Values \pm 95% Confidence Region for Growth of <i>Scenedesmus obliquus</i> with Arsenic at Its Given Concentration Versus the Control with No Metals.....	38
10	Mean Values \pm 95% Confidence Region for Lipid Analysis of <i>Scenedesmus obliquus</i> with Arsenic at Its Given Concentration Versus the Control with No Metals.....	39
11	Mean Values \pm 95% Confidence Region for Growth of <i>Scenedesmus obliquus</i> with All Metals at 1/20, 1/10, 1/4, and 1/2 Concentration Versus the Control with No Metals	42

12	Mean Values \pm 95% Confidence Region for Lipid Analysis of <i>Scenedesmus obliquus</i> with All Metals at 1/20, 1/10, 1/4, and 1/2 Concentration Versus the Control with No Metals	44
13	Estimated Concentrations of Metals in Liquid Media (mg/L) Using Various Literature Sources and Assumptions	48
14	Concentration of Each Metal That Was Beneficial to the Algae.....	56
15	Individual Metals at 1/20 the Reference Concentrations and Their Effect on the Algae	57

LIST OF FIGURES

Figure		Page
1	Cyanotech Corporation facility in Kona, Hawaii [29].	7
2	Raceway photo-bioreactor design [26].	10
3	Schematic representation of airlift (A) and bubble column (B) reactors [34].	11
4	Schematic representation of horizontal tubular reactor with a degassing unit, composed of parallel sets of tubes (A) or a loop tube (B) [34].	13
5	Schematic representation of helical tubular reactors: biocoil (A) and conical framework (B) [34].	14
6	Tube reactors and experimental setup.	18
7	Reactors used to grow inocula.	19
8	Linear regression between OD and TSS with uncertainty region.	27
9	Growth of <i>Scenedesmus obliquus</i> with zinc, lead, cobalt, and chromium at their given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * \rightarrow represents significant difference from control determined by Tukey's test for that point forward.	29
10	Lipid analysis for <i>Scenedesmus obliquus</i> with zinc, lead, cobalt, and chromium at their given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point, the symbol * \rightarrow represents significant difference from control determined by Tukey's test for that point forward.	31
11	Growth of <i>Scenedesmus obliquus</i> with mercury, nickel, copper, and cadmium at their given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point, the symbol * \rightarrow represents significant difference from control determined by Tukey's test for that point forward.	32
12	Lipid analysis for <i>Scenedesmus obliquus</i> with mercury, nickel, copper, and cadmium at their given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point.	34

13	Growth of <i>Scenedesmus obliquus</i> with selenium at its given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point.	36
14	Lipid analysis for <i>Scenedesmus obliquus</i> with selenium at its given concentration versus the control with no metals, mean values \pm SD plotted.	37
15	Growth of <i>Scenedesmus obliquus</i> with arsenic at its given concentration versus the control with no metals, mean values \pm SD plotted.	38
16	Lipid analysis for <i>Scenedesmus obliquus</i> with arsenic at its given concentration versus the control with no metals, mean values \pm SD plotted.	39
17	Summary of results for experiments with individual metals at the reference concentrations, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test.....	41
18	Growth of <i>Scenedesmus obliquus</i> with all metals at 1/20, 1/10, 1/4, and 1/2 the reference concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point, the symbol *→ represents significant difference from control determined by Tukey's test for that point forward.....	42
19	Lipid analysis for <i>Scenedesmus obliquus</i> with all metals at 1/20, 1/10, 1/4, and 1/2 the reference concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point, the symbol *→ represents significant difference from control determined by Tukey's test for that point forward.	44
20	Summary of results for experiments with all metals at different concentrations, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test.....	45
21	Comparison of growth between polystyrene and borosilicate reactors, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test.....	50
22	Growth comparison between <i>Scenedesmus obliquus</i> and <i>Neochloris oleabundans</i> with selenium.....	53
23	Lipid analysis comparison between <i>Scenedesmus obliquus</i> and <i>Neochloris oleabundans</i> with selenium.....	54

INTRODUCTION

The United States generates about 5.8×10^9 metric tons of CO_2 per year. Electricity generation accounts for 41% of that amount or 2.4×10^9 metric tons per year [1]. These numbers will only increase as the demand for energy rises. There is much debate on the effects that rising CO_2 concentrations will have on the environment, but there is a general desire in the energy community to create technology that will have less of a carbon footprint.

CO_2 emitted from smoke stacks can be captured by a physical or chemical absorption and then transported to somewhere it can be stored, usually underground or deep under the ocean. Capturing the CO_2 in this manner would reduce the efficiency of the power plants by 10-30% which would increase the cost of electricity by 30-130% [2].

One method that may be commercially feasible is using microalgae for carbon sequestration. Algae need light, carbon dioxide, and nutrients (such as nitrogen and phosphorous) to grow. As CO_2 is often a limiter for growth, algae growth could be enhanced by growing the algae on the flue gas emitted by coal plants. This serves a dual purpose in that the algae are sequestering the CO_2 and in turn could produce biodiesel and other products. Many studies have shown that microalgae grow well with high concentrations of CO_2 that are found in flue gas [3-8]. Algae have also been shown to produce far more fuel per land area than any other biological organism [9-11]. The dry biomass can also be used in applications such as co-firing [12].

In addition to high amounts of CO_2 , flue gas also contains several other chemical species such as sulfur oxides (referred to as SO_x and comprising mostly SO_2 and SO_3), nitrogen oxides (referred to as NO_x and comprising mostly NO and NO_2) [13], heavy

metal species including Ag, As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Se, and Zn [14, 15], and carbon compounds including polycyclic aromatic hydrocarbons (PAHs) [16].

Many researchers have studied growing algae on flue gas. However, limited studies are available regarding what effect heavy metals in the flue gas have on the algae. The purpose of this study was to understand how metals might impact algae that will be used for biodiesel. Specifically, the factors determining how much biodiesel can be produced include the growth of the algae and the accumulation of lipids within the algae.

It should be noted that the term “heavy metals” does not have a set definition. In general a heavy metal is a member of elements that exhibit metallic properties, mainly transition metals and some metalloids, but the parameters for the definition have not been set and definitions, therefore, vary. For the purpose of this thesis the term “heavy metals” includes the metals and metalloids that were tested, namely: As(III), Cd, Cr(VI), Co, Cu, Pb, Ni, Hg, Se, and Zn.

LITERATURE REVIEW

How CO₂ levels affect the environment

The sun powers the Earth's weather and climate. Radiation from the sun is predominantly in the visible or near-visible (ultraviolet) part of the spectrum (42% visible [0.4-0.75 μ m], 12% UV [0.01-0.4 μ m], 44% IR [0.75-3 μ m]). About one-third of the solar energy that reaches the Earth's atmosphere is reflected directly back to space. The remaining solar energy is absorbed by the surface and atmosphere of the Earth. The Earth's surface is warmed and re-radiates energy back to space. Because the Earth is colder than the sun, it radiates energy at much longer wavelengths (0% [0.01-0.75 μ m], 0.005% [0.75-3 μ m], remaining [3 μ m+]) [17, 18].

The longer wavelength makes it harder for the energy to pass through certain gases in our atmosphere called "greenhouse gases" and escape back into space. As much of this radiation cannot escape back into space it is re-radiated back to Earth and further warms the surface. This process is called the greenhouse effect.

Water vapor is the most prominent greenhouse gas in our atmosphere, followed by carbon dioxide [17]. Besides these gases, ozone, aerosols, and other trace gases in our atmosphere also have a greenhouse effect [19].

Carbon dioxide is the gas of greatest importance for "global warming" because the amount of carbon burned from fossil fuels is 400 times the net primary productivity (NPP) of the planet's current biota [20]. This means that the amount of carbon released to the atmosphere from fossil fuel in one year will take 400 years to remove by natural processes. The result is an ever increasing concentration of CO₂ in our atmosphere. The

Mauna Loa observatory in Hawaii has been recording atmospheric levels of CO₂ for 50 years, and in that time, CO₂ concentrations have risen from about 310 parts per million (ppm) to about 390 ppm [21]. Higher CO₂ levels will continue to raise the surface temperature of the Earth. This will also have a compounding effect as more water will be vaporized into the atmosphere with the higher temperature.

The increase in temperature, as well as other factors like the increase in acidity of water environments due to absorbing more CO₂, could damage fragile ecosystems [22]. To prevent this, it will be critical to manage the large carbon footprint that is being created by industrial processes.

Algae could utilize waste CO₂ and produce biodiesel

Flue gas from fossil fuels contains from 3 to 15% CO₂ depending on the carbon content of the fuel and the amount of excess air necessary for the combustion process [23]. This is much higher than atmospheric CO₂ levels. There are many organisms that can utilize CO₂ for growth and could therefore be used to absorb flue gas emissions, such as higher plants, submerged higher plants and seaweed, and microbes including algae. Higher plants grown in open air can be enhanced with fertilization from the flue gas; however the only possible delivery system for the flue gas would be a series of distribution pipes and productivities of the plants would not justify such a system. Using greenhouses to grow the higher plants could also not be justified. Submerged plants and seaweed will exhibit low productivities unless there is significant turbulence that is needed for good water exchange in dense stands of submerged plants, and creation of such turbulence is not practical. In addition, all microbes except for microalgae require some inorganic reducing agent (H₂, H₂S, NH₃, pyrites, etc.) Microalgae, therefore, appear

to be the only viable solution [24]. Algae growth rates can increase by injection of flue gas directly into the growth media; CO₂ that would otherwise be pumped into the atmosphere would be absorbed into the algae. Increased algae yields could then be used to create renewable fuels.

The concept of using CO₂ in power plant flue gases to grow microalgae was first studied by Golueke and Oswald over 50 years ago [25]. They used municipal sewage ponds injected with flue gas to grow the algae and harvested the algae by settling. Instead of biodiesel production they digested the algae to produce methane and used the recycled digester residue as nutrients. They successfully demonstrated this system on a laboratory scale.

From 1978 to 1996 the U.S. Department of Energy's Office of Fuels Development funded a large program known as the Aquatic Species Program (ASP). The main focus of the ASP was the production of biodiesel from high lipid-content algae grown in ponds using waste CO₂ from coal fired power plants. The program made many large breakthroughs.

The program was able to collect about 3,000 strains of organisms, and after screening, isolation and characterization was able to narrow the strains down to around 300 that had a very good ability to produce natural oils as well as the ability to grow under severe conditions such as extremes in temperature, pH and salinity.

The program heavily researched nutrient deficiency and the "lipid trigger" in which microorganisms produce more lipids with deficiency of nutrients like nitrogen. They found that with nitrogen deficiency the algae do produce more lipids, but that

overall more lipids are not produced due to diminishes in algae growth that also occur with nutrient deficiency.

The program made headway in metabolic engineering by isolating the gene that is responsible for oil production in algae and over-expressing that gene in order to produce more oils.

The program made large efforts to establish the feasibility of large-scale algae production in open ponds. They found that careful control of pH and other physical conditions allowed for over 90% CO₂ consumption of flue gas that was pumped into the ponds. Test sites were set up in California, Hawaii, and New Mexico. Attempts to achieve consistently high productivities of algal biomass were hampered by temperature drops during the night in New Mexico.

Cost analysis for large scale microalgae production was also improved with this project. They found that factors that influence cost the most are biological, and not engineering related. Therefore the largest obstacle to be overcome is finding or producing an organism that can convert the sun's energy on near-theoretical levels. Even with aggressive assumptions they found the cost of biodiesel from microalgae to be two times higher than current petroleum costs (in 1998) [26].

Another large program was started in Japan in 1990 in order to produce a system for fixation of flue gas by microalgae. The research for this program was done by the Research Institute of Innovative Technology for the Earth (RITE). Much like ASP, a large part of the program focused on selection of strains particularly suited for fixation of CO₂ and production of high value products. In addition, biotechnology for gene

manipulation of the algae was studied. A large part of the program focused on development of a highly-effective photo-bioreactor for fixation of flue gas CO₂ [27, 28].

After much research and development no commercial large scale facility has been produced to fixate flue gas CO₂ and produce biofuels. Cyanotech Corporation, however, has developed a large scale commercial facility for production of nutritional products. Their facility in Kona, Hawaii cultures algae strains *Spirulina* and *Haematococcus pluvialis*. A small power plant supplies power for raceway paddle wheels and the flue gas CO₂ is used to enhance algae growth. A picture of this facility is shown in Figure 1. Cost of commercial algae produced in this facility is \$5,000 per ton. The allowable cost for renewable fuels production could be no more than \$250 per ton [29].



Figure 1. Cyanotech Corporation facility in Kona, Hawaii [29].

Many other researchers have studied the use of flue gas to enhance microalgae growth and they have all encountered the same problems. The requirements for large areas of land, favorable climates and ample water supplies will restrict the potential of this technology. Additionally, even with favorable assumptions the cost of microalgae-fuels is still high [30]. Much more research and development will need to be done, and in the end algae products that have a higher value than fuels may need to be created.

Biodiesel and bioethanol are potential renewable fuels. Two proposed sources of these biofuels are agricultural crops and microalgae. Biofuels produced from crops using existing methods will never be able to replace fossil-based transport fuels, but biodiesel from microalgae has the potential to completely displace petroleum-derived transport fuels. In addition, microalgae can be grown without affecting supply of food and other crop-based products [11].

There are a few reasons why algae are a better option than crop-based renewable fuels. Oil content within the plants that is used to make biodiesel is much higher in microalgae than in oil crops. Some microalgae have oil content that can reach 80% of the dry weight of the algae. While this is an extreme case it is quite common for algae to have lipid concentrations ranging from 20-50% of dry mass [10]. Oil based crops such as soybean and oil palm that are currently being used to create biodiesel usually have an oil concentration of less than 5% of dry biomass [11]. The end result is that algae can produce far more biodiesel per amount of land used than oil crops.

Another advantage of microalgae based biodiesel is that it does not compete with food crop. Microalgae grow in aqueous media and therefore do not require fertile agricultural land for growth. In 2008 the United States Department of Agriculture

(USDA) released a report showing that world market prices for major food commodities such as grains and vegetable oils had risen sharply to over 60% of what they were two years earlier. One of the main reasons for this is an increase in the demand for biofuels feedstock [31]. This is a major argument for microalgae based biofuels as any increase in plant based biofuels will also mean a decrease in the land available for food crops and therefore major increases in food prices.

Producing algae on a large scale to absorb flue gas CO₂

To be able to absorb flue gas CO₂, algae will need to be produced on a large scale. Any method used for production of microalgae on a large scale must accomplish a few things. Sunlight must be delivered to the algae. The algae need to be mixed and aerated in order to prevent settling, low yields, unstable algal populations and difficulty distributing nutrients [32]. Nutrients needed for the algae to grow must be added. Flue gas or other forms of CO₂ should be pumped into the algae.

Large-scale production of algae will also require that the algae can be economically produced. There are only two types of practical methods for large-scale production. These are raceways and tubular photo-bioreactors [10].

The most common method proposed for use in large scale production of microalgae is the raceway. The raceway design has a paddle that sends the algae and media around a track; this allows the algae to be mixed. Media and nutrients as well as CO₂ are added as the algae go around the track. The top of the raceway is exposed to the atmosphere and therefore it is an “open” design. A rough model of the raceway design is shown in Figure 2 [26]. An open design like the raceway is the most likely to be used for

large scale production as closed designs are too expensive for application to low-cost production systems [29].

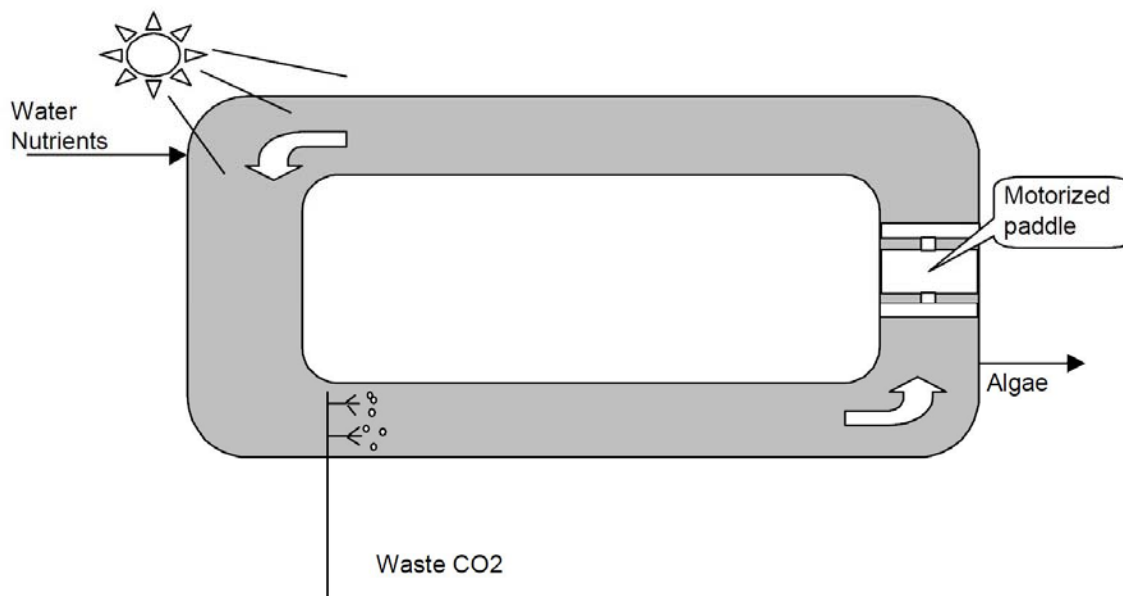


Figure 2. Raceway photo-bioreactor design [26].

While low cost is an advantage of raceways they do have many disadvantages as well. In raceways the only cooling that can be achieved is from evaporation. Evaporative water losses can be significant. Carbon dioxide losses to the open atmosphere can also be significant and raceways will be far less efficient in large scale production than closed reactors. Productivity is also affected by contamination with native microorganisms that are more adapted to the climate than high oil-yielding strains. The biomass concentrations remain low for raceways due to poor mixing [10]. Efforts are being made to improve mixing and aeration in raceways. This will increase CO₂ consumption, which will enhance algal biomass growth [33].

The only types of closed reactors that could be used for large scale production are tube reactors. Tube reactors could conceivably come in any configuration, but there are three main types: vertical, horizontal, and helical [34].

Vertical tube reactors are the simplest of all of the designs. They are sometimes called airlift or bubble column reactors because gas is bubbled from the bottom of the reactors. This provides adequate mixing for the microalgae, and can be used to supply CO_2 to the algae as well as efficiently remove O_2 . A simple representation of this type of reactor is shown in Figure 3 [34]. Plastics and glasses are the most common materials

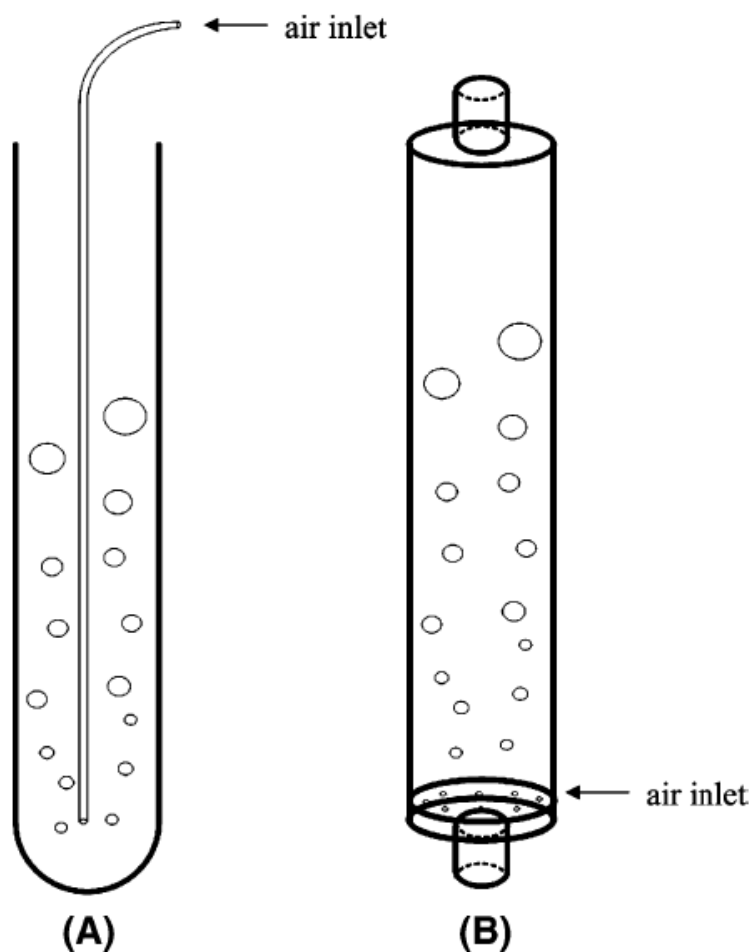


Figure 3. Schematic representation of airlift (A) and bubble column (B) reactors [34].

used for these types of reactors. Both of these materials are commonly available and therefore non-expensive. Polyethylene tubing can be used to create “bag” type reactors. These reactors have been used for outdoor cultivation of algae at Utah State University. The advantage of bag reactors is their particularly low cost. However, the plastic is flexible and the bags can be problematic in that they can tear very easily. Rigid vertical tube reactors made of different kinds of plastics and glass have also been widely used.

Horizontal tubular reactors have become more popular recently. These reactors generally contain a light harvesting section, where the algae flow through the horizontal tubes and are exposed to sunlight, and a reservoir connected via an airlift pump for degassing. A diagram of this type of reactor is shown in Figure 4 [34]. Systems like these have been found to be efficient and cost effective for lab scale reactors. The length of the light harvesting tubes are limited as dissolved oxygen levels can rise to a level that will inhibit photosynthesis [11]. However, this problem can easily be solved by adding more tube sets to the degasser instead of making the tubes longer. Each tube set will have its own airlift pump as the pump is a simple and efficient way to remove oxygen through the degasser [35]. This also should mean that scale up will pose no problems, as a single degasser unit can be connected to many tube sets. One big drawback to this design is the amount of land area required and cost for scale up, as producing large amounts of algae in the narrow tubes will require a huge amount of tubes that will need to be placed horizontally.

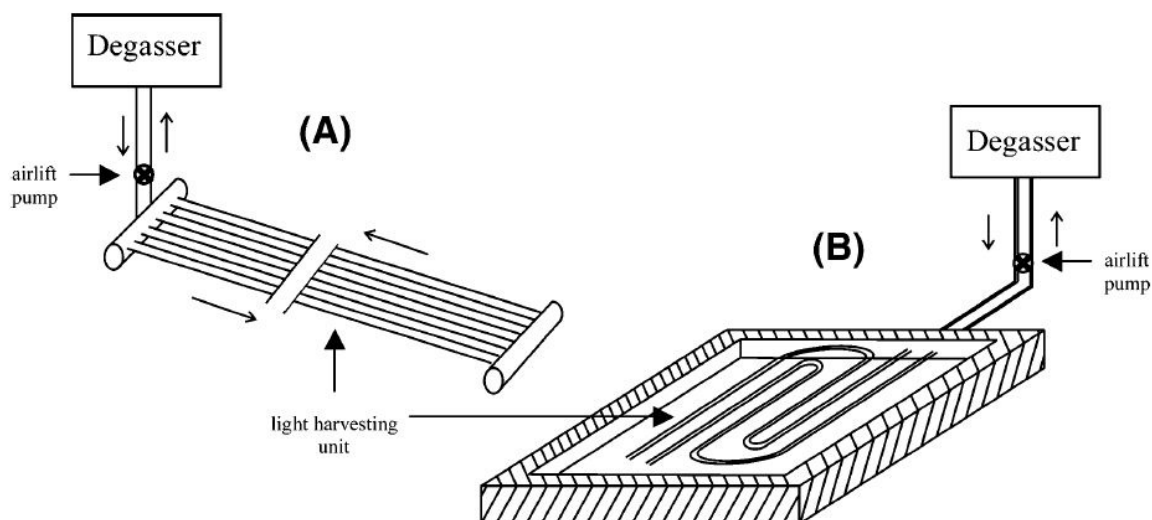


Figure 4. Schematic representation of horizontal tubular reactor with a degassing unit, composed of parallel sets of tubes (A) or a loop tube (B) [34].

The last common type of tube reactor is the helical reactor. As with the horizontal tube reactor, long lengths of closed tubing require a degassing unit to get rid of excess oxygen. Instead of using straight tubing that is aligned horizontally or vertically the tubing is coiled in an open circular framework. The reactor also includes a centrifugal pump to drive the culture broth and a heat exchange system. Figure 5 shows two common configurations for helical reactors, biocoil and conical framework [34].

Chemical species found in flue gas and their effect on algae

In addition to high amounts of CO_2 , flue gas also contains other chemical species that might not have such a positive effect on the algae as the CO_2 .

NO_x and SO_x are gases found in small concentrations in flue gas. One study found that adding 100 ppm NO and NO_2 to a simulated flue gas with 10% CO_2 had no effect on the growth of *Chlorella sp. HA-1*, but that same strain did not tolerate 50 ppm SO_2 [3].

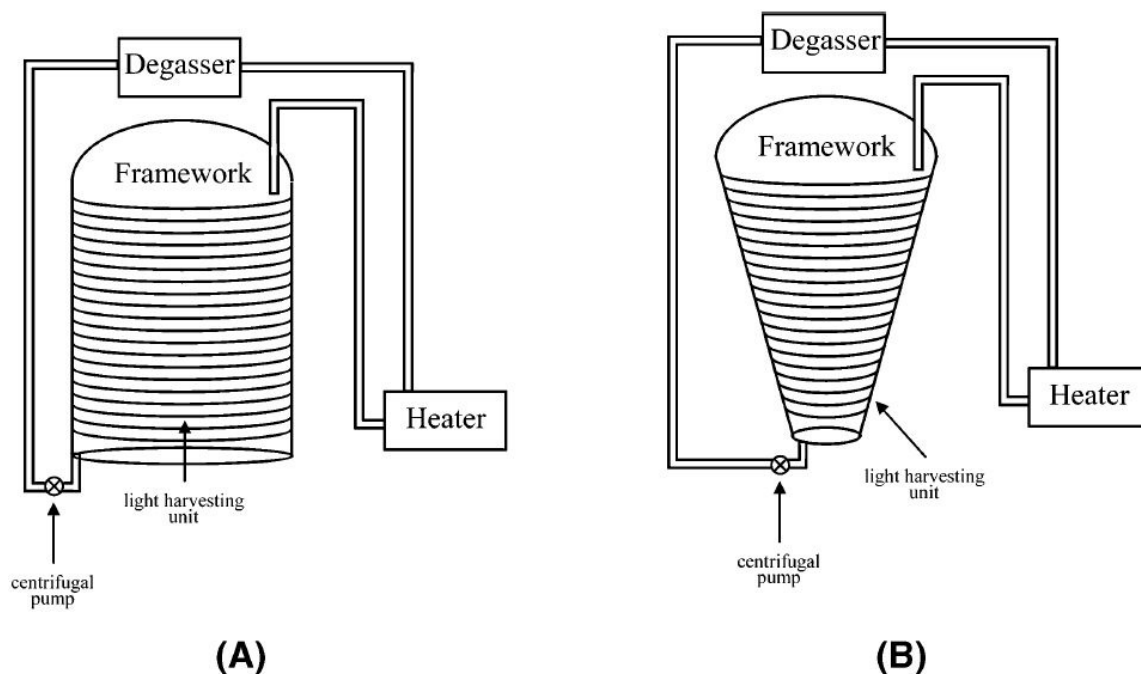


Figure 5. Schematic representation of helical tubular reactors: biocoil (A) and conical framework (B) [34].

Another study used a simulated flue gas with 15% CO₂ and found that *Chlorella sp. T-1* tolerated 20ppm SO_x and 60 ppm NO_x [4]. Both of the experiments were conducted at a constant flow rate of simulated flue gas. The algae might have tolerated a higher concentration of SO_x and NO_x if a pH control system had been connected to the simulated flue gas, as higher concentrations of SO_x and NO_x turn the media more acidic.

Effects that heavy metals in flue gas could have on the algae

While high concentrations of CO₂ in flue gas will be beneficial to the algae, heavy metals found in the flue gas might not. Heavy metals could have several effects on algae. First of all, it is important to understand that algae have an immense capability to sorb metals when initially exposed to them, and then after prolonged exposure to uptake the metals into the cells [36]. Some metals at low concentration are crucial for nutrition in

algae. For example, copper, iron, zinc, and manganese act as important cofactors for many enzymes and are essential for both mitochondrial and chloroplast functions in plants [37]. Also, at low concentrations algae can overcome the effect of highly reactive metal species through different defense mechanisms like antioxidants [38]. At higher concentrations metals become toxic and will damage the algal cells [37, 38]. This would probably result in slow growth and less lipid production. In addition to fuels, algae have been used as nutritional products [29] and high metal concentrations in the algae would also make them unsuitable to be used for human or animal consumption.

The effect of heavy metals in flue gas on algae for the purpose of biofuels production has not been widely researched, however, one study measured the concentrations of several different metals from flue gas that was used to grow a red macro alga called *Gracilaria cornea*. The flue gas was taken from a power plant in Ashkelon, Israel. The seaweed was also grown with commercial CO₂ for comparison. The growth of *G. cornea* was equally enhanced with flue gas and commercial CO₂. The concentrations of many different heavy metals in the media and in the tissue and agar of the seaweed were monitored. Concentrations were low and many fell below the sensitivity of the instrument used. There was little difference between the metal concentrations in the tissue and agar for the seaweed grown with flue gas and the seaweed grown with commercial CO₂. This likely explains why there was no difference in growth between the two. The concentration of metals in the biomass also met European standards for human and animal consumption [39].

Another study observed the effect of Cu²⁺, Zn²⁺, and Pb²⁺ on the fresh water plant *Hydrilla verticillata*. This experiment did not involve flue gas. The plants were incubated

in 100 μ M solutions of metal nitrates for 10 days. Copper ions suppressed lipid metabolism in the plant, whereas zinc and lead ions induced the accumulation of biomass and elevated the content of some phospholipids and glycolipids [40].

EXPERIMENTAL SETUP AND PROCEDURES

Reactors used for experiments

The reactors that were used for these experiments were glass tube reactors as shown in Figure 6. The reactors are approximately 1.1 liters in volume. Light was supplied to the reactors with a bank of fluorescent lights directly behind the reactors. The reactors were made of borosilicate glass. These “airlift” type reactors were well-mixed by air bubbles pumped into the bottom through glass capillary tubes. CO₂ was also pumped through the capillary tubes in order to keep the reactors between pH 6.9 and 7.5. To be able to monitor pH and make sure it stayed within the allotted range, pH was measured three times each week during an experiment, and CO₂ flow was adjusted accordingly. CO₂ flowrate for all 15 reactors started off at approximately 25 ml/min and was approximately 100 ml/min by the end of the experiment. A 50 ml syringe that was connected to a silicone tube at the top was used to extract samples from the reactors. The end of the tube was 2 inches from the bottom of the reactor. This is an ideal location to extract samples from the reactor as mixing is good at this location, and is high enough from the bottom of the reactor to not be affected by settling.

Inoculation procedure

Scenedesmus obliquus was the algae strain used for all of the experiments. Algae were first grown on petri dishes in order to maintain and verify that the algae were not contaminated.

When starting a new experiment, algae from the petri dishes were used to inoculate 3L polystyrene reactors as shown in Figure 7. Light was continuously supplied

to the reactors and the pH was controlled to 7 ± 0.1 . This allowed the algae to grow fast and after approximately one week the reactors had grown enough algae to start the tube reactors at about 0.8 g/L.

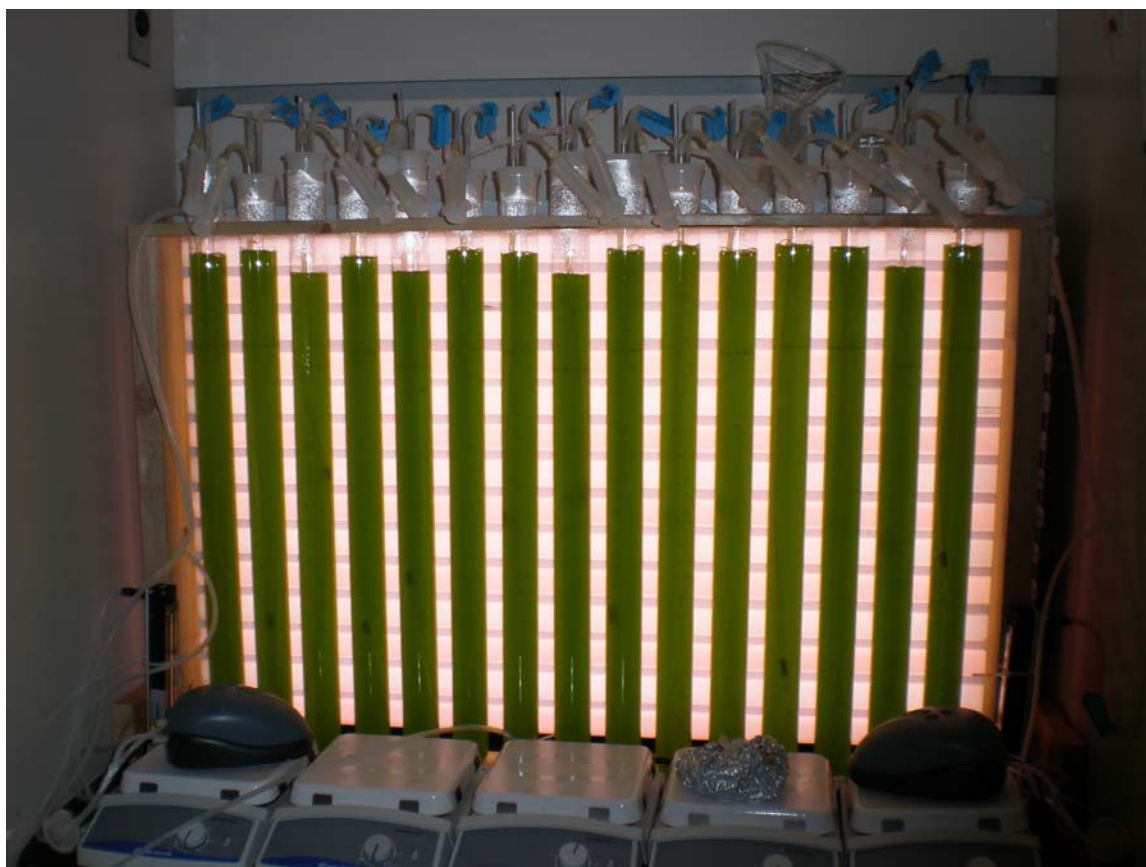


Figure 6. Tube reactors and experimental setup.

When the algae had grown enough to start the new reactors at 0.8 g/L they were centrifuged. To keep from damaging the algae, the centrifuge was set to only exert about 3900xg on the algae. The algae were then washed and re-suspended in new media and then centrifuged again. This was done to remove any chelating agents produced by the algae during the original growth cycle [41]. The algae were then re-suspended, their optical density was measured, and they were added to the new reactors so that the density of the algae in the new reactors was about 0.8 g/L.

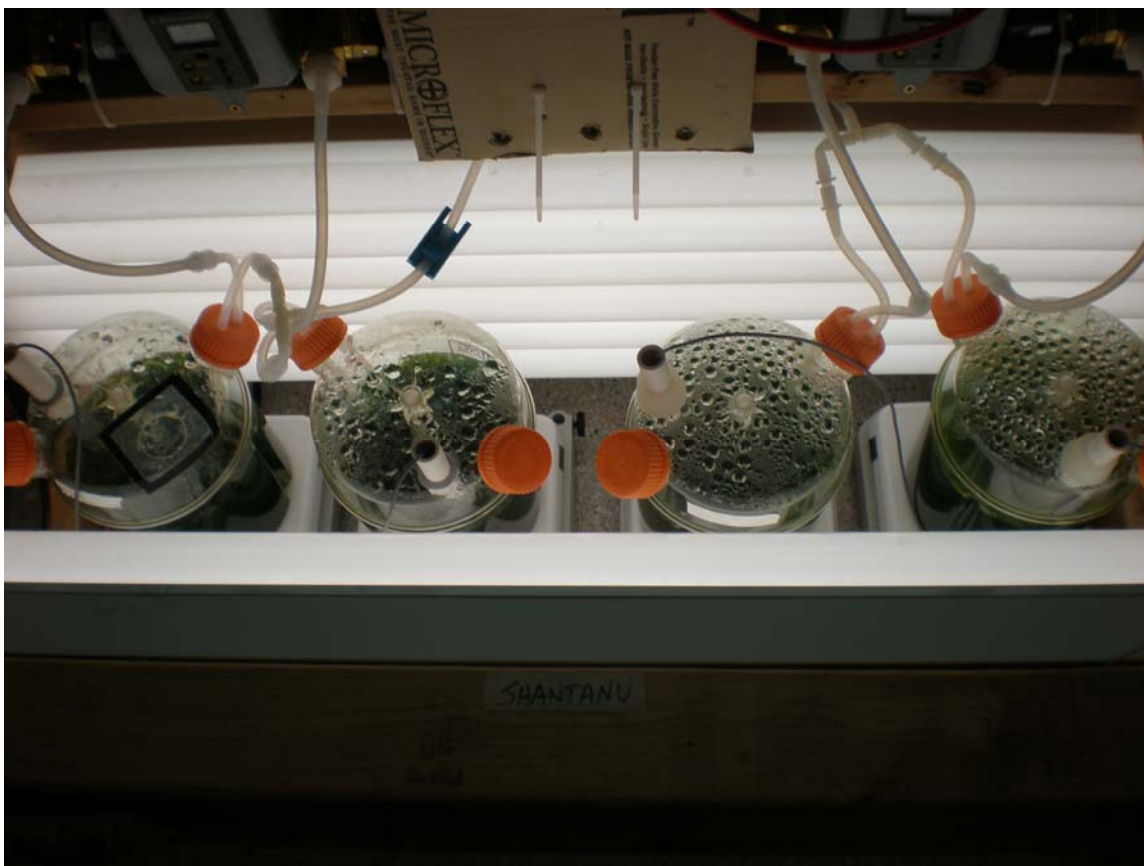


Figure 7. Reactors used to grow inocula.

Media used

The media used to grow *Scenedesmus obliquus* was APS freshwater medium. APS refers to Arizona Public Service, the company from which the media originated (see Appendix A). The media contained three stock solutions: Fe-EDTA, micronutrient, and macronutrient. These were all added together when growing the inocula. However, during the metals experiments the Fe-EDTA stock solution was left out as the EDTA is a chelator and will bind with the metals, and therefore could interfere with the interaction between the metals and the algae.

Determination of metals concentration

The second column of Table 1 shows the highest mass fractions of different heavy metals (mg metal/kg fly ash) [42]. It should be noted that the term “fly ash” refers to the portion of total ash that becomes airborne when burning coal. The reference concentrations of the heavy metals that were used in the experiments are also shown in Table 1 in the last column. There are many assumptions made for the calculations in Table 1 because comprehensive metal concentration data for uncaptured fly ash is lacking and quite variable. All assumptions used were to give the highest concentration possible and are as follows:

- a.** Carbon content in coal is usually 60-80% [43] and 60% will be used (60% gives a higher concentration). It is also assumed that all carbon converts to CO₂
- b.** Ash content of coal is usually 0.1-20% [44, 45] and 20% will be used (20% gives a higher concentration). Also, depending on furnace design, up to 80% of total ash is fly ash [46] and usually only 1% of fly ash enters reactors due to removal systems [47]
- c.** Sparge rate of CO₂ is about 0.006 vvm (vessel volume per minute; this is approximately what we have observed at USU)
- d.** Metals in fly ash are completely leached into the media
- e.** Growth cycle is 14 days (Approximate growth cycle for this strain of algae)
- f.** Metal accumulates each time the water is recycled and the water will be recycled 20 times.

Heavy metals were added to the media as metal salt solutions immediately before the reactors were inoculated. The Mathcad file with all of the calculations for metal concentrations will be included in Appendix B.

Table 1. Highest Mass Fractions [42] and Reference Metal Concentrations Calculated from Literature (Appendix B)

Component	Mass Fraction (mg/kg)	Est. Conc. In Liquid Media (mg/L)
Arsenic	391.0	1.56
Cadmium	76.0	0.30
Chromium	651.0	2.60
Cobalt	79.0	0.32
Copper	655.0	2.62
Lead	273.0	1.09
Nickel	1270.0	5.08
Mercury	49.5	0.20
Selenium	49.5	0.20
Zinc	2200.0	8.80

Sampling

Immediately after reactors had been inoculated for a new experiment, samples were taken. To measure pH and growth, 3 ml was extracted from the reactor. To get enough biomass for lipid analysis, 45 ml was extracted from the reactor.

During the first three days pH was measured every day as the growing algae would dramatically change the amount of CO₂ needed. Samples were also taken three times a week during the experiments, usually on Mondays, Wednesdays, and Fridays. Growth and pH were measured on all three days and lipid analysis samples were taken on Mondays and Fridays. The experiments were usually about three weeks in length.

Measurement of growth with OD and conversion from OD to TSS

Total suspended solids (TSS) is a measurement of dry mass in the reactor and is reported in g/L. This is how the growth was reported. Growth of the algae was measured using optical density (OD). This method is used because it is quick and easy, and can be used to compare the growth of the algae grown with metals against the control that is grown without metals. However, OD cannot be converted to TSS unless a correlation has been made.

To be able to develop this correlation, an experiment was conducted for seven days. Florescent light was supplied to 3L polystyrene reactors 24 hours a day so that the algae would grow rapidly. Every day, six samples were acquired from the reactor for OD and six samples were acquired for TSS. The OD was measured with a Thermo Electron Corporation Genesys 5 spectrophotometer at both 680nm and 750nm. It was discovered that 750nm gives a better linear correlation. TSS was measured according to Standard Methods for the Examination of Water and Wastewater [48]. (The protocol DOC316.53.001204 from the Hach Company was followed for TSS analysis.)

The resulting correlation that was used to convert all OD measurements to TSS in g/L is almost perfectly linear and is shown in Equation (1). The uncertainty of the correlation will be discussed in a later section.

$$TSS = 0.4585(OD_{750nm}) + 0.0116 \quad (1)$$

Transesterification

To do lipid analysis, 45 ml was sampled from the reactors twice a week. It was centrifuged so that the algae could collect in the bottom of the vial and be separated from the supernatant. The remaining biomass was lyophilized to remove any remaining moisture. The completely dry mass was ground into a powder and the sample was transesterified.

Transesterification is a process by which lipids (saturated fatty acids (SFAs) and poly unsaturated fatty acids (PUFAs)) extracted from organisms such as algae are converted into fatty acid methyl esters (FAMES), which can be used as biodiesel. Toxic solvents such as hexane or chloroform are used to extract the lipids that the algae have accumulated, and then transesterification occurs separately using an acidified methanol solution. A single-step reactive extraction method that combines the sequential extraction followed by transesterification using acidified methanol was used to determine the lipid content of the algae. This method is known as *in situ* transesterification and was developed by Daniel Nelson at Utah State University [49]. A copy of the standard operating procedure for the method is included in Appendix C. The transesterified samples were analyzed on an Agilent Technologies 7890A gas chromatograph system using a 14 point FAME calibration mix from Supelco.

Statistical analysis

Statistical analysis for growth and lipid analysis was conducted using a two-way ANOVA followed by Tukey's test. Two-way ANOVA was used to determine if any of

the results were significantly different than the control for an entire experiment. A built-in function for Microsoft Excel was used to do the two-way ANOVA.

If the two-way ANOVA determined that there was a significant difference in the experiment, Tukey's test was then used to further determine significant difference.

Tukey's test uses the results from an ANOVA analysis and a studentized range distribution to compare means and determine if they are significantly different [50].

Published values for the studentized range distribution are shown in Table 2 [50].

The variable $\alpha = 0.05$ refers to a 95% confidence interval, the variable R refers to the number of means being compared, and the variable v_2 refers to the degree of freedom. For the experiments conducted the means being compared were the mean of the controls conducted in triplicate, and the means of the different metal conditions conducted in triplicate. The full two-way ANOVA for each experiment has too many means to compare to be able to use the table, therefore, a two-way ANOVA was calculated for each day samples were taken, and those values were used for Tukey's test. The significant difference from Tukey's test will be shown on the figures of the results. The calculations will not be included in this report.

Uncertainty analysis

An uncertainty analysis was also conducted for the growth and lipid accumulation results to determine the 95% confidence region. For both growth and lipid accumulation the general equation used to determine the 95% confidence interval is shown in Equation (2).

Table 2. Studentized range distribution used for Tukey's test [50].

R = Range (Number of Groups)														
v2	2	3	4	5	6	7	8	9	10	12	14	16	18	20
6	3.46	4.34	4.90	5.30	5.63	5.90	6.12	6.32	6.49	6.79	7.03	7.24	7.43	7.59
	5.24	6.33	7.03	7.56	7.97	8.32	8.61	8.87	9.10	9.10	9.48	10.08	10.32	10.54
7	3.34	4.16	4.68	5.06	5.36	5.61	5.82	6.00	6.16	6.43	6.66	6.85	7.02	7.17
	4.95	5.92	6.54	7.01	7.37	7.68	7.94	8.10	8.37	8.71	9.00	9.24	9.46	9.65
8	3.26	4.04	4.53	4.89	5.17	5.40	5.60	5.77	5.92	6.18	6.39	6.57	6.73	6.87
	4.75	5.64	6.20	6.62	6.96	7.24	7.47	7.68	7.86	8.18	8.44	8.66	8.85	9.03
9	3.20	3.95	4.41	4.76	5.02	5.24	5.43	5.59	5.74	5.98	6.19	6.36	6.51	6.64
	4.60	5.43	5.96	6.35	6.66	6.91	7.13	7.33	7.49	7.78	8.03	8.23	8.41	8.57
10	3.15	3.88	4.33	4.65	4.91	5.12	5.30	5.46	5.60	5.83	6.03	6.19	6.34	6.47
	4.48	5.27	5.77	6.14	6.43	6.67	6.87	7.05	7.21	7.49	7.71	7.91	8.08	8.23
11	3.11	3.82	4.26	4.57	4.82	5.03	5.20	5.35	5.49	5.71	5.90	6.06	6.20	6.33
	4.39	5.15	5.62	5.97	6.25	6.48	6.67	6.84	6.99	7.25	7.47	7.65	7.81	7.95
12	3.08	3.77	4.20	4.51	4.75	4.95	5.12	5.27	5.39	5.62	5.80	5.95	6.09	6.21
	4.32	5.05	5.50	5.84	6.10	6.32	6.51	6.67	6.81	7.06	7.27	7.44	7.59	7.73
13	3.06	3.73	4.15	4.45	4.69	4.88	5.05	5.19	5.32	5.53	5.71	5.86	6.00	6.11
	4.26	4.96	5.40	5.73	5.98	6.19	6.37	6.53	6.67	6.90	7.10	7.27	7.42	7.55
14	3.03	3.70	4.11	4.41	4.64	4.83	4.99	5.13	5.25	5.46	5.64	5.79	5.92	6.03
	4.21	4.89	5.32	5.63	5.88	6.08	6.26	6.41	6.54	6.77	6.96	7.13	7.27	7.40
15	3.01	3.67	4.08	4.37	4.59	4.78	4.94	5.08	5.20	5.40	5.57	5.72	5.85	5.96
	4.17	4.84	5.25	5.56	5.80	5.99	6.16	6.31	6.44	6.66	6.85	7.00	7.14	7.26
16	3.00	3.65	4.05	4.33	4.56	4.74	4.90	5.03	5.15	5.35	5.52	5.66	5.79	5.90
	4.13	4.79	5.19	5.49	5.72	5.92	6.08	6.22	6.35	6.56	6.74	6.90	7.03	7.15
17	2.98	3.63	4.02	4.30	4.52	4.70	4.86	4.99	5.11	5.31	5.47	5.61	5.73	5.84
	4.10	4.74	5.14	5.43	5.66	5.85	6.01	6.15	6.27	6.48	6.66	6.81	6.94	7.05
18	2.97	3.61	4.00	4.28	4.49	4.67	4.82	4.96	5.07	5.27	5.43	5.57	5.69	5.79
	4.07	4.70	5.09	5.38	5.60	5.79	5.94	6.08	6.20	6.41	6.58	6.73	6.85	6.97
19	2.96	3.59	3.98	4.25	4.47	4.65	4.79	4.92	5.04	5.23	5.39	5.53	5.65	5.75
	4.05	4.67	5.05	5.33	5.55	5.73	5.89	6.02	6.14	6.34	6.51	6.65	6.78	6.89
20	2.95	3.58	3.96	4.23	4.45	4.62	4.77	4.90	5.01	5.20	5.36	5.49	5.61	5.71
	4.02	4.64	5.02	5.29	5.51	5.69	5.84	5.97	6.09	6.29	6.45	6.59	6.71	6.82
24	2.92	3.53	3.90	4.17	4.37	4.54	4.68	4.81	4.92	5.10	5.25	5.38	5.44	5.59
	3.96	4.55	4.91	5.17	5.37	5.54	5.69	5.81	5.92	6.11	6.26	6.39	6.51	6.61
30	2.89	3.49	3.85	4.10	4.30	4.46	4.60	4.72	4.82	5.00	5.15	5.27	5.38	5.48
	3.89	4.45	4.80	5.05	5.24	5.40	5.54	5.65	5.76	5.93	6.08	6.20	6.31	6.41
40	2.86	3.44	3.79	4.04	4.23	4.39	4.52	4.63	4.73	4.90	5.04	5.16	5.27	5.36
	3.82	4.37	4.70	4.93	5.11	5.26	5.39	5.50	5.60	5.76	5.90	6.02	6.12	6.21
60	2.83	3.40	3.74	3.98	4.16	4.31	4.44	4.55	4.65	4.81	4.94	5.06	5.15	5.24
	3.76	4.28	4.59	4.82	4.99	5.13	5.25	5.36	5.45	5.60	5.73	5.84	5.93	6.02
120	2.80	3.36	3.68	3.92	4.10	4.24	4.36	4.47	4.56	4.71	4.84	4.95	5.04	5.13
	3.70	4.20	4.50	4.71	4.87	5.01	5.12	5.21	5.30	5.44	5.56	5.66	5.75	5.83
∞	2.77	3.31	3.63	3.86	4.03	4.17	4.29	4.39	4.47	4.62	4.74	4.85	4.93	5.01
	3.64	4.12	4.40	4.60	4.76	4.88	4.99	5.08	5.16	5.29	5.40	5.49	5.57	5.65

Note: Studentized range q distribution table of critical values for $\alpha = .05$ and $\alpha = .01$

$$U = \sqrt{\left(t_{95} \frac{S}{\sqrt{n}}\right)^2 + s^2 + b^2} \quad (2)$$

In Equation (2) the variable U stands for the total uncertainty. The first term under the square root is the 95% confidence interval for the standard deviation of each triplicate

of samples, where S stands for the standard deviation. This is a random uncertainty. The variable s stands for any other random uncertainties that are present, and the variable b stands for any systematic uncertainties that are present.

For the growth results the random and systematic uncertainties were determined from a linear regression uncertainty analysis between OD and TSS [51]. The calculations for the linear regression uncertainty analysis will not be included in this report. The final uncertainty is shown in Equation (3). Figure 8 shows the graph of the uncertainty equation with the linear regression and original points.

$$U_{TSS} = \sqrt{0.001097OD_{750nm}^2 - 0.007254OD_{750nm} + 0.017117} \quad (3)$$

Equation (3) varies from 0.07 g/L to 0.12 g/L. To be conservative 0.12 g/L will be used for the total uncertainty from the linear regression between OD and TSS.

For the lipid accumulation results a detailed uncertainty analysis was used to determine the random and systematic uncertainties [51]. The calculations for this uncertainty analysis will not be included in this report. The final values for the total random and systematic uncertainties respectively are: $s = 0.007345 \text{ kg FAME/kg Algae}$ and $b = 0.000833 \text{ kg FAME/kg Algae}$.

The 95% confidence intervals will be shown in tables following the result plots.

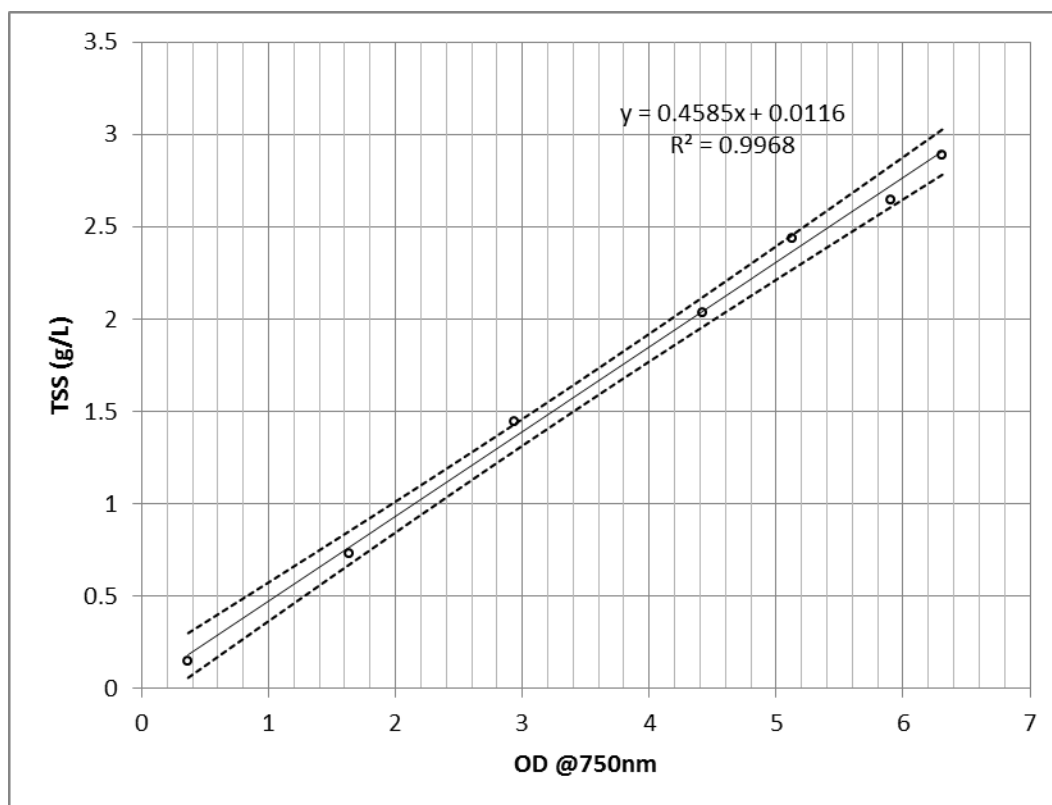


Figure 8. Linear regression between OD and TSS with uncertainty region.

RESULTS

Introduction of results

The plots contained in this section show the growth and lipid accumulation of the algae with different individual metals and with all metals at different concentrations. Selenium was the first metal tested, and the experiment was grown for two weeks. After that the experiments were lengthened to three weeks, as the algae were still growing at the end of two weeks. Measurements were plotted every two or three days over the entire growth. Growth is plotted as TSS in grams of algae per liter of reactor. Lipid analysis is plotted as kg FAME/kg algae, or in other words the amount of FAME produced per amount of dry weight of the algae. The individual points of each plot are connected to help visualize the results.

Zinc, lead, cobalt, and chromium

The metals zinc, lead, cobalt, and chromium were all tested in the same experiment and therefore have the same control and were analyzed with the same Two-Way ANOVA. The results for all of these metals are presented together. Figure 9 shows the growth curve for all of these metals together. The average values with the standard deviation (SD) are shown as the experiment was conducted in triplicate. The results from Tukey's test are also shown in Figure 9. Table 3 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the growth.

As can be seen from Figure 9 zinc, lead, cobalt, and chromium at their given concentrations all have a negative effect on the growth of the algae. The density of the algae with lead was close to the density of the control at the beginning, but by day six the

density was significantly lower than the control. After that not only was the density of the algae grown with lead significantly lower than the control, the density started decreasing. The density of the algae with the other metals was significantly lower than the control after the fourth day. The density stayed significantly lower for the remainder of the experiment, but the algae with cobalt were still able to grow at a reasonable rate. The reactors with zinc and cobalt demonstrated minimal growth.

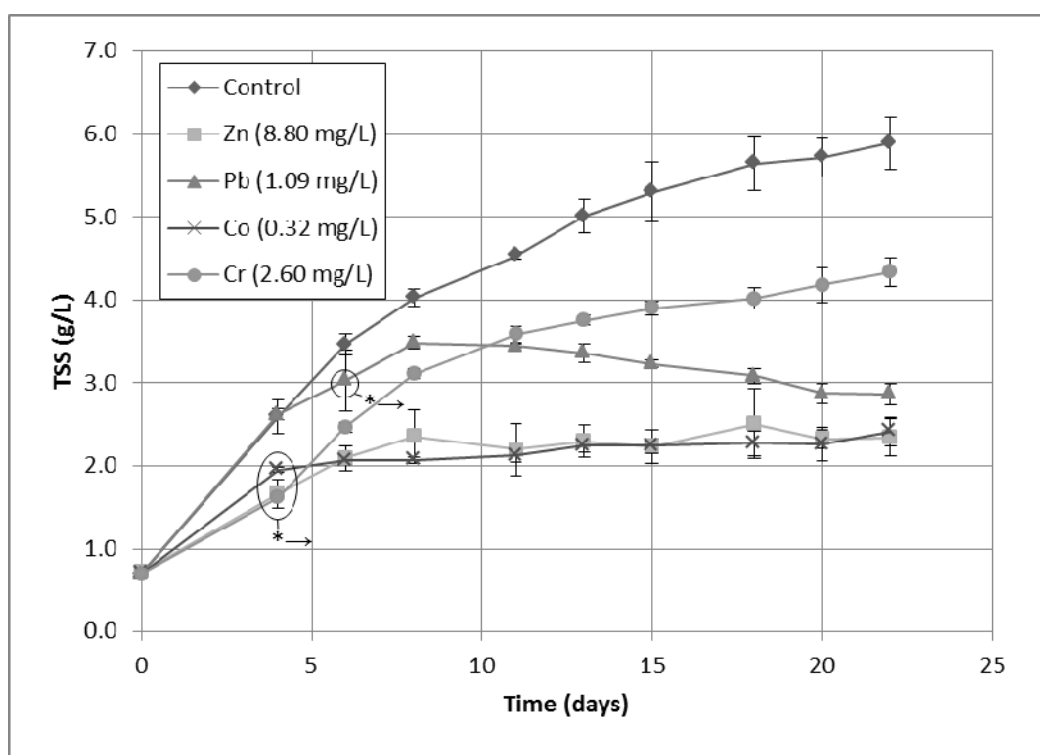


Figure 9. Growth of *Scenedesmus obliquus* with zinc, lead, cobalt, and chromium at their given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * \rightarrow represents significant difference from control determined by Tukey's test for that point forward.

Table 3. Mean Values \pm 95% Confidence Region for Growth of *Scenedesmus obliquus* with Zinc, Lead, Cobalt, and Chromium at Their Given Concentrations Versus the Control with No Metals

TSS (g/L)	day									
control/metal	0	4	6	8	11	13	15	18	20	22
control	0.70 ± 0.12	2.59 ± 0.53	3.46 ± 0.34	4.03 ± 0.28	4.54 ± 0.16	5.01 ± 0.52	5.30 ± 0.90	5.64 ± 0.80	5.72 ± 0.58	5.89 ± 0.80
Zn (8.80 mg/L)	0.72 ± 0.12	1.66 ± 0.45	2.09 ± 0.39	2.35 ± 0.81	2.20 ± 0.79	2.30 ± 0.50	2.24 ± 0.52	2.51 ± 1.06	2.33 ± 0.30	2.34 ± 0.56
Pb (1.09 mg/L)	0.69 ± 0.12	2.62 ± 0.21	3.03 ± 0.93	3.48 ± 0.23	3.45 ± 0.14	3.36 ± 0.29	3.23 ± 0.17	3.08 ± 0.26	2.87 ± 0.32	2.86 ± 0.34
Co (0.32 mg/L)	0.70 ± 0.12	1.95 ± 0.14	2.06 ± 0.17	2.07 ± 0.15	2.12 ± 0.22	2.24 ± 0.21	2.24 ± 0.25	2.28 ± 0.39	2.26 ± 0.51	2.41 ± 0.43
Cr (2.60 mg/L)	0.69 ± 0.12	1.62 ± 0.12	2.47 ± 0.19	3.10 ± 0.19	3.58 ± 0.28	3.76 ± 0.20	3.90 ± 0.23	4.02 ± 0.35	4.18 ± 0.53	4.34 ± 0.45

Figure 10 shows the lipid analysis curve for the experiment with the metals zinc, lead, cobalt and chromium. The average values with standard deviation are shown as the experiment was conducted in triplicate. The results from Tukey's test are also shown in Figure 10. Table 4 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the lipid analysis.

Most of the metals in Figure 10 caused a decrease in the accumulation of lipids in the algae. For zinc, which is the metal at the highest concentration of any of the metals tested, lipid content was significantly lower than the control from the beginning and stayed that way. Cobalt showed a similar pattern, but not as extreme. Lipid content was significantly lower than the control after day eight. Lipid content for chromium was significantly lower than the control from the beginning and stayed that way, but did not do as poorly as zinc and cobalt. Lipid content was significantly higher than the control for lead at day eight, but overall, there was no significant difference from the control.

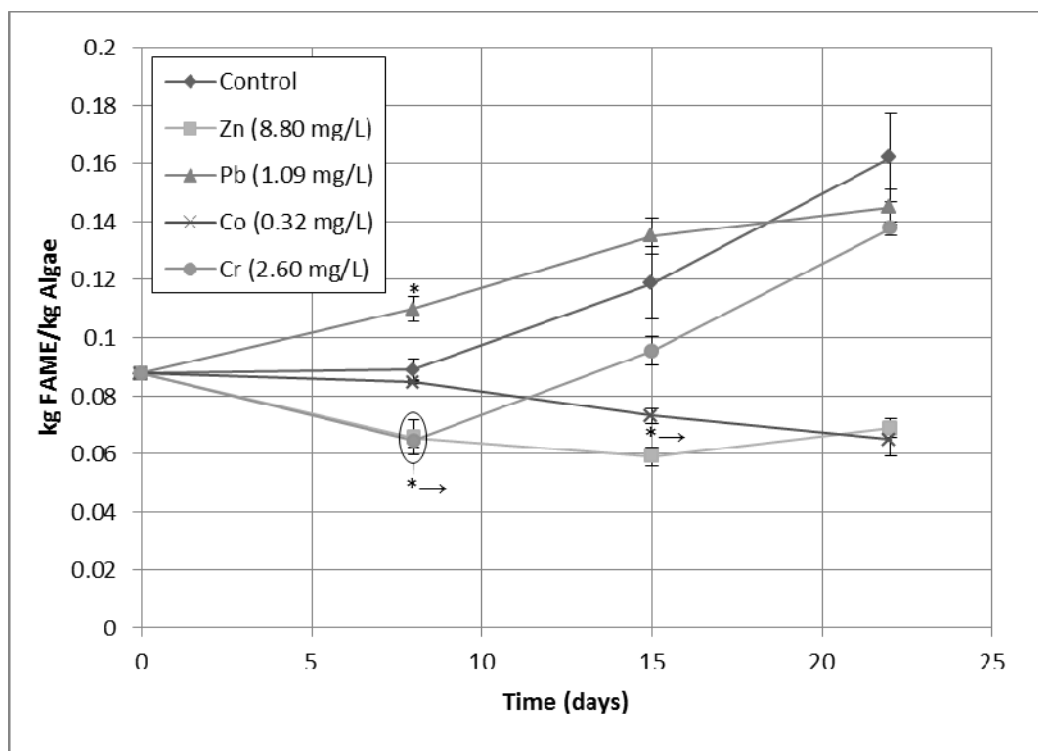


Figure 10. Lipid analysis for *Scenedesmus obliquus* with zinc, lead, cobalt, and chromium at their given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point, the symbol *→ represents significant difference from control determined by Tukey's test for that point forward.

Table 4. Mean Values \pm 95% Confidence Region for Lipid Analysis of *Scenedesmus obliquus* with Zinc, Lead, Cobalt, and Chromium at Their Given Concentrations Versus the Control with No Metals

kg FAME/ kg Algae	day			
control/metal	0	8	15	22
control	0.0876 ± 0.0074	0.0890 ± 0.0118	0.1189 ± 0.0319	0.1621 ± 0.0389
Zn (8.80mg/L)	0.0876 ± 0.0074	0.0657 ± 0.0167	0.0592 ± 0.0109	0.0688 ± 0.0110
Pb (1.09 mg/L)	0.0876 ± 0.0074	0.1010 ± 0.0131	0.1351 ± 0.0171	0.1450 ± 0.0167
Co (0.32 mg/L)	0.0876 ± 0.0074	0.0846 ± 0.0075	0.0731 ± 0.0098	0.0650 ± 0.0162
Cr (2.60 mg/L)	0.0876 ± 0.0074	0.0645 ± 0.0090	0.0954 ± 0.0143	0.1377 ± 0.0095

Mercury, nickel, copper, and cadmium

The metals mercury, nickel, copper, and cadmium were all tested in the same experiment and are presented together. Figure 11 shows the growth curve for all of these metals together. The average values with standard deviation are shown as the experiment was conducted in triplicate. The results from Tukey's test are also shown in Figure 11. Table 5 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the growth.

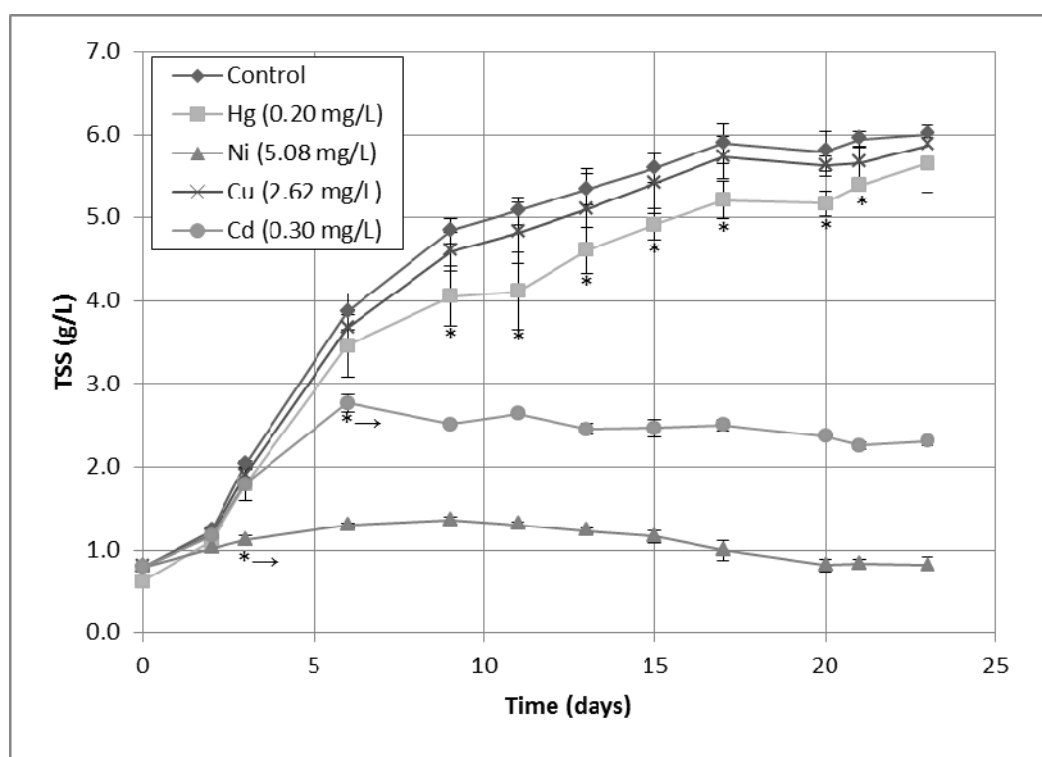


Figure 11. Growth of *Scenedesmus obliquus* with mercury, nickel, copper, and cadmium at their given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point, the symbol * \rightarrow represents significant difference from control determined by Tukey's test for that point forward.

As can be seen from Figure 11, nickel and cadmium had drastic effects on the growth of the algae. Mercury had a small effect, and copper had no noticeable effect. The

effect of nickel was the most severe. After day three the density of the algae with nickel was significantly lower than the control. The density of the algae with nickel hardly increased at all and even started to decrease after day nine. The concentration of nickel eventually killed the algae; on day 23 the culture turned brown. Cadmium showed a similar pattern as nickel, only less extreme. After day six, the density became significantly different than the control, and density started decreasing. Mercury caused only a slight decrease in the growth of the algae. The density of the algae with mercury was significantly lower than the control after the sixth day, but only slightly. The mercury was at a very low concentration. At higher concentrations it would no doubt have a more drastic effect. There was no significant difference at any point for the density of the algae with copper compared to the control.

Table 5. Mean Values \pm 95% Confidence Region for Growth of *Scenedesmus obliquus* with Mercury, Nickel, Copper, and Cadmium at Their Given Concentrations Versus the Control with No Metals

TSS (g/L)	day											
control/metal	0	2	3	6	9	11	13	15	17	20	21	23
control	0.79 ± 0.14	1.23 ± 0.12	2.03 ± 0.17	3.87 ± 0.56	4.84 ± 0.38	5.08 ± 0.42	5.34 ± 0.48	5.59 ± 0.44	5.89 ± 0.61	5.80 ± 0.60	5.94 ± 0.27	6.01 ± 0.16
Hg (0.20 mg/L)	0.62 ± 0.19	1.10 ± 0.23	1.79 ± 0.48	3.46 ± 0.94	4.05 ± 0.90	4.12 ± 1.15	4.61 ± 0.70	4.92 ± 0.50	5.21 ± 0.57	5.17 ± 0.75	5.39 ± 0.15	5.65 ± 0.90
Ni (5.08 mg/L)	0.79 ± 0.12	1.02 ± 0.12	1.11 ± 0.18	1.30 ± 0.14	1.35 ± 0.17	1.31 ± 0.14	1.23 ± 0.14	1.16 ± 0.22	1.00 ± 0.33	0.82 ± 0.22	0.83 ± 0.18	0.82 ± 0.29
Cu (2.62 mg/L)	0.79 ± 0.12	1.19 ± 0.14	1.90 ± 0.29	3.67 ± 0.58	4.59 ± 0.59	4.82 ± 0.93	5.10 ± 1.2	5.41 ± 0.92	5.72 ± 0.66	5.62 ± 0.31	5.66 ± 0.49	5.87 ± 0.63
Cd (0.30 mg/L)	0.79 ± 0.14	1.17 ± 0.15	1.79 ± 0.13	2.77 ± 0.29	2.52 ± 0.13	2.64 ± 0.13	2.45 ± 0.21	2.47 ± 0.28	2.50 ± 0.22	2.37 ± 0.14	2.25 ± 0.16	2.31 ± 0.18

Figure 12 shows the lipid analysis curve for the experiment with the metals mercury, nickel, copper and cadmium. The average values with standard deviation are shown as the experiment was conducted in triplicate. The results from Tukey's test are

also shown in Figure 12. Table 6 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the lipid analysis.

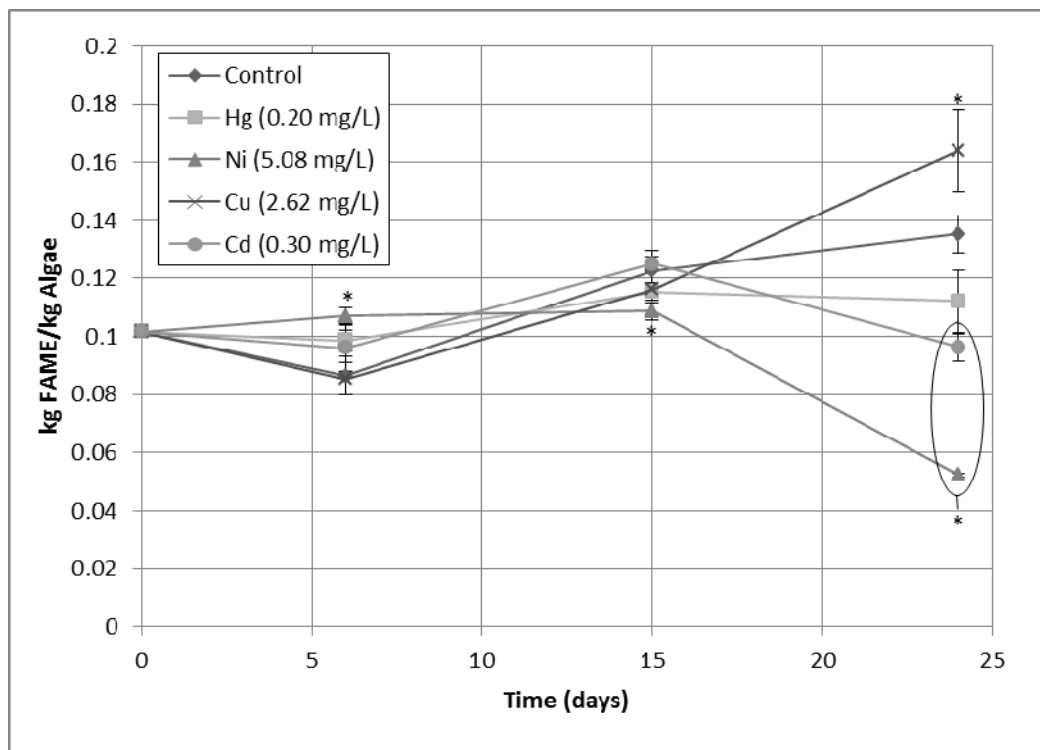


Figure 12. Lipid analysis for *Scenedesmus obliquus* with mercury, nickel, copper, and cadmium at their given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point.

As can be seen from Figure 12 the metals used in the experiment did not have very much effect on the accumulation of lipids inside the cell, except for the very last day. Nickel, the metal that killed the algae, caused a significantly higher production of lipids than the control on the sixth day, but after that lipid content started becoming significantly lower and on day 24 the lipid content plummeted. Lipid content for mercury and cadmium were significantly lower than the control only on day 24, and lipid content for copper was significantly higher than the control only on day 24.

Table 6. Mean Values \pm 95% Confidence Region for Lipid Analysis of *Scenedesmus obliquus* with Mercury, Nickel, Copper, and Cadmium at Their Given Concentrations Versus the Control with No Metals

kg FAME/ kg Algae	day			
	0	6	15	24
control	0.1016 ± 0.0074	0.0864 ± 0.0178	0.1227 ± 0.0192	0.1352 ± 0.0180
Hg (0.20 mg/L)	0.1016 ± 0.0074	0.0987 ± 0.0117	0.1153 ± 0.0105	0.1122 ± 0.0277
Ni (5.08 mg/L)	0.1016 ± 0.0074	0.1071 ± 0.0105	0.1087 ± 0.0104	0.0522 ± 0.0076
Cu (2.62 mg/L)	0.1016 ± 0.0074	0.0853 ± 0.0157	0.1163 ± 0.0080	0.1640 ± 0.0356
Cd (0.30 mg/L)	0.1016 ± 0.0074	0.0961 ± 0.0213	0.1250 ± 0.0096	0.0962 ± 0.0140

Selenium

The metal selenium was tested in its own experiment, so this metal will be presented by itself. Figure 13 shows the growth curve for selenium. The average values with standard deviation are shown as the experiment was conducted in triplicate. The results from Tukey's test are also shown in Figure 13. Table 7 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the growth.

As can be seen from Figure 13 selenium only had a slight negative effect on the growth of the algae. The growth was significantly lower only on the sixth and eighth days. The concentration was really low, and with a higher concentration selenium would probably have a more drastic effect on the algae.

Figure 14 shows the lipid analysis curve for the experiment with selenium. The average values with standard deviation are shown as the experiment was conducted in triplicate. The results from Tukey's test are also shown in Figure 14. Table 8 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the lipid analysis.

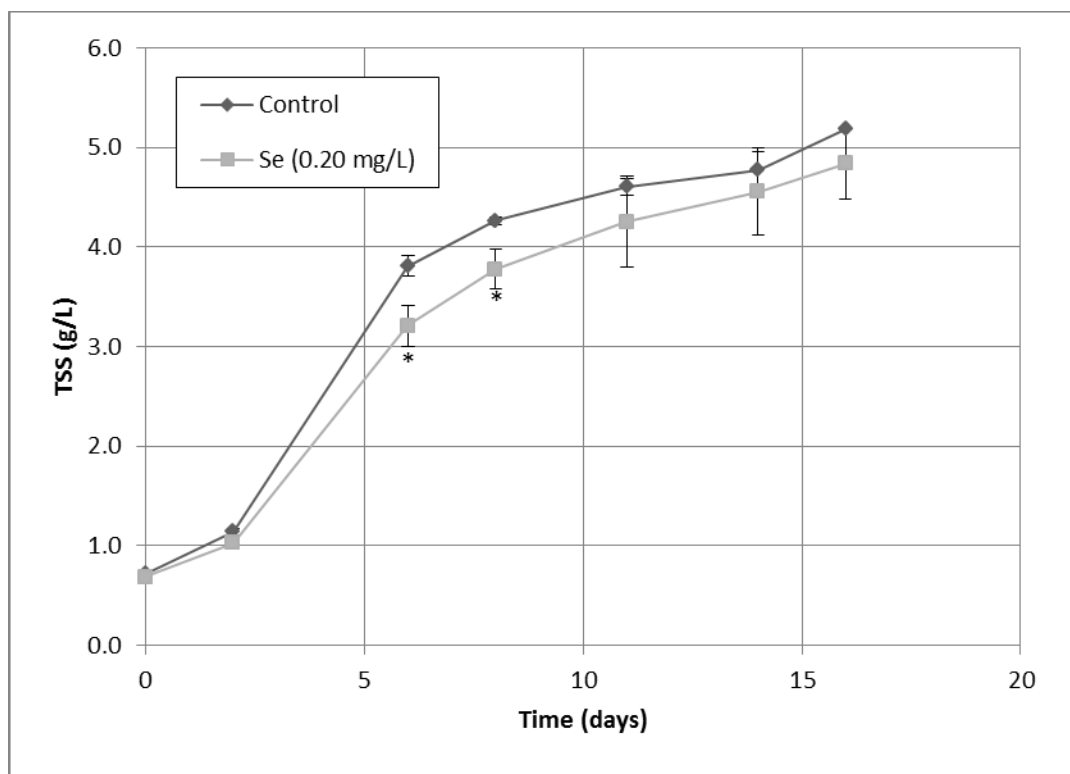


Figure 13. Growth of *Scenedesmus obliquus* with selenium at its given concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point.

Table 7. Mean Values \pm 95% Confidence Region for Growth of *Scenedesmus obliquus* with Selenium at Its Given Concentration Versus the Control with No Metals

TSS (g/L)	day						
control/metal	0	2	6	8	11	14	16
control	0.72 ± 0.12	1.14 ± 0.15	3.81 ± 0.28	4.26 ± 0.16	4.61 ± 0.24	4.77 ± 0.46	5.18 ± 0.12
Se (0.20 mg/L)	0.69 ± 0.13	1.03 ± 0.16	3.21 ± 0.52	3.77 ± 0.51	4.25 ± 1.13	4.56 ± 1.09	4.84 ± 0.91

As can be seen from Figure 14 selenium at its given concentration had no effect on the accumulation of lipids within the algae.

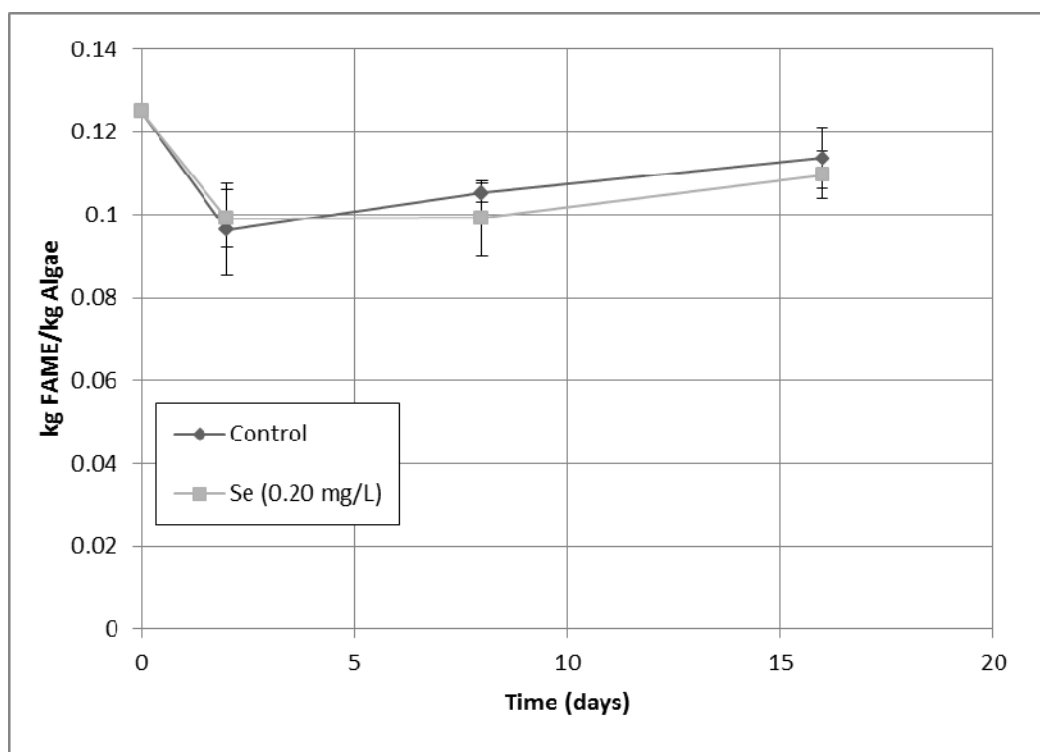


Figure 14. Lipid analysis for *Scenedesmus obliquus* with selenium at its given concentration versus the control with no metals, mean values \pm SD plotted.

Table 8. Mean Values \pm 95% Confidence Region for Lipid Analysis of *Scenedesmus obliquus* with Selenium at Its Given Concentration Versus the Control with No Metals

kg FAME/ kg Algae	day			
	0	2	8	16
control	0.1249 ± 0.0074	0.0964 ± 0.0288	0.1052 ± 0.0092	0.1137 ± 0.0198
Se (0.20 mg/L)	0.1249 ± 0.0074	0.0991 ± 0.0185	0.0992 ± 0.0234	0.110 ± 0.0160

Arsenic

The metal arsenic was tested in its own experiment, so this metal will be presented by itself. Figure 15 shows the growth curve for arsenic. The average values with standard deviation are shown as the experiment was conducted in triplicate. The

results from Tukey's test are also shown in Figure 15. Table 9 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the growth.

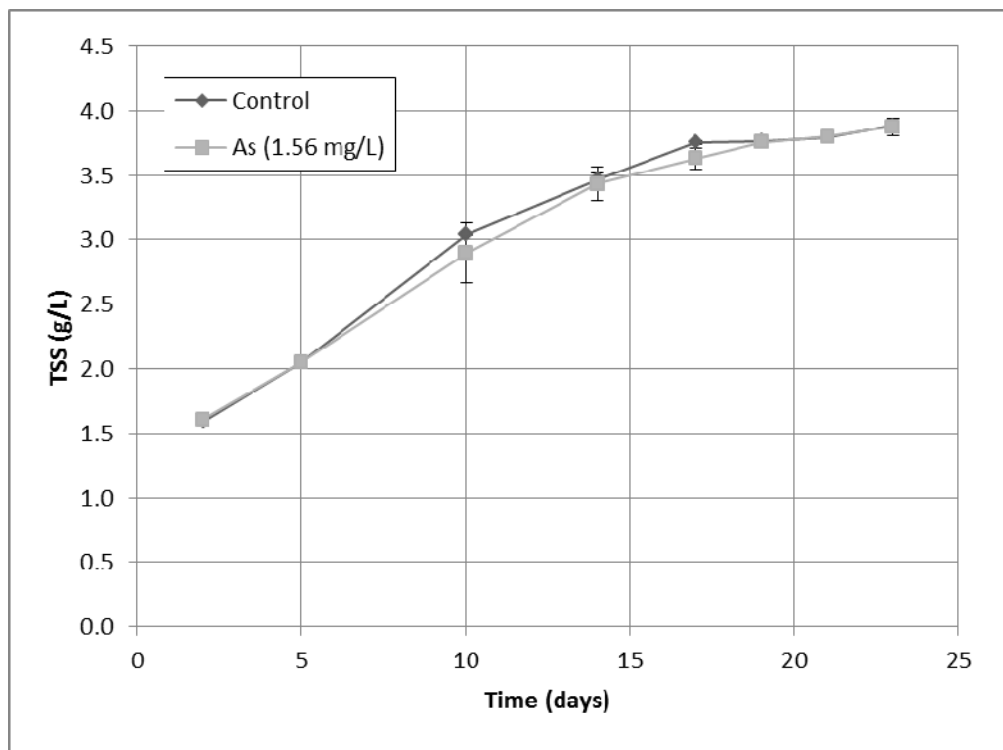


Figure 15. Growth of *Scenedesmus obliquus* with arsenic at its given concentration versus the control with no metals, mean values \pm SD plotted.

Table 9. Mean Values \pm 95% Confidence Region for Growth of *Scenedesmus obliquus* with Arsenic at Its Given Concentration Versus the Control with No Metals

TSS (g/L)	day							
control/metal	2	5	10	14	17	19	21	23
control	1.59 ± 0.14	2.05 ± 0.4	3.04 ± 0.13	3.46 ± 0.49	3.75 ± 0.17	3.76 ± 0.19	3.79 ± 0.23	3.88 ± 0.52
As (1.56 mg/L)	1.61 ± 0.25	2.05 ± 0.27	2.90 ± 2.09	3.43 ± 1.18	3.63 ± 0.75	3.76 ± 0.36	3.80 ± 0.15	3.88 ± 0.61

Figure 16 shows the lipid analysis curve for the experiment with the metal arsenic. The average values with standard deviation are shown as the experiment was conducted in triplicate. The results from Tukey's test are also shown in Figure 16. Table

10 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the lipid analysis.

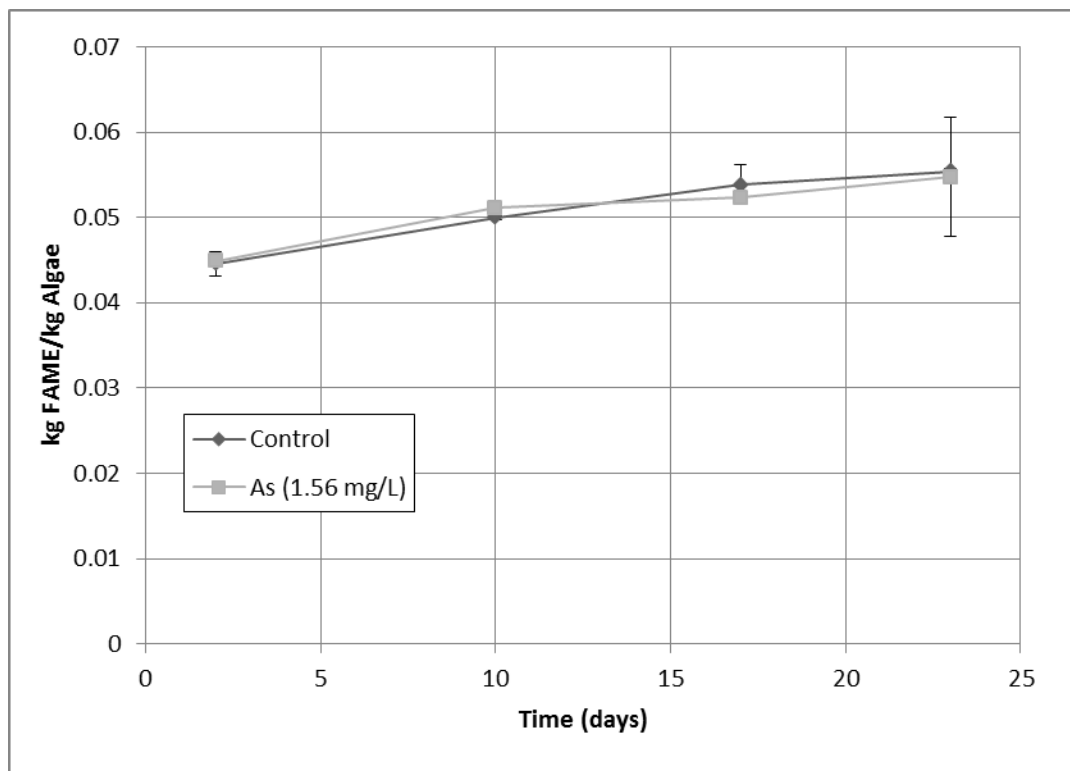


Figure 16. Lipid analysis for *Scenedesmus obliquus* with arsenic at its given concentration versus the control with no metals, mean values \pm SD plotted.

Table 10. Mean Values \pm 95% Confidence Region for Lipid Analysis of *Scenedesmus obliquus* with Arsenic at Its Given Concentration Versus the Control with No Metals

kg FAME/ kg Algae	day			
	2	10	17	23
control	0.0446 ± 0.0082	0.0500 ± 0.0074	0.0538 ± 0.0094	0.0554 ± 0.0074
As (1.56 mg/L)	0.0449 ± 0.0074	0.0511 ± 0.0075	0.0523 ± 0.00757	0.0548 ± 0.0189

As can be seen from Figures 15 and 16 arsenic at its given concentration had no effect on growth or lipid accumulation in the algae.

Summary of individual metals

The first set of experiments was conducted to see the effect of each metal individually at the reference concentrations as shown in Table 1. To summarize the results for all of the individual metals tested, the area under the approximate curves (the individual points with connected lines) for all plots was calculated. By calculating the area under the approximate curves, the average effect of each of the metals over the entire length of the experiment can be shown. To normalize all the results and be able to present them together, the area under the control curves was set as 100 percent and the area under the other curves was compared as a percentage of the control curves. Figure 17 shows the area under the curves for the experiments with each individual metal. The first column for the bar graph shows the percentages of the area under the curves for growth, the second column shows the percentages of the area under the curves for lipid analysis, and the last column shows the percentages of biodiesel yield, which are the percentages from the first two columns multiplied together. Standard deviations are given for all values. The results from Tukey's test are also shown in Figure 17. Figure 17 includes the results from four experiments, and each one of these have a control with a different standard deviation. The standard deviations shown for the controls are the largest out of the four different experiments. The results are ordered from highest total lipids produced to lowest total lipids produced.

As can be seen from Figure 17 all of the metals at their given concentrations decrease the growth of the algae at least somewhat, and many of them dramatically reduce the growth. Lipid content within the algae seems to be less affected by the metals. The lipid accumulation for many of the metals was close to, and even greater than, the

lipid content of the controls for some metals. However, many of the metals also resulted in a reduction in lipid accumulation, and combined with the growth the biodiesel yield was always less than the controls.

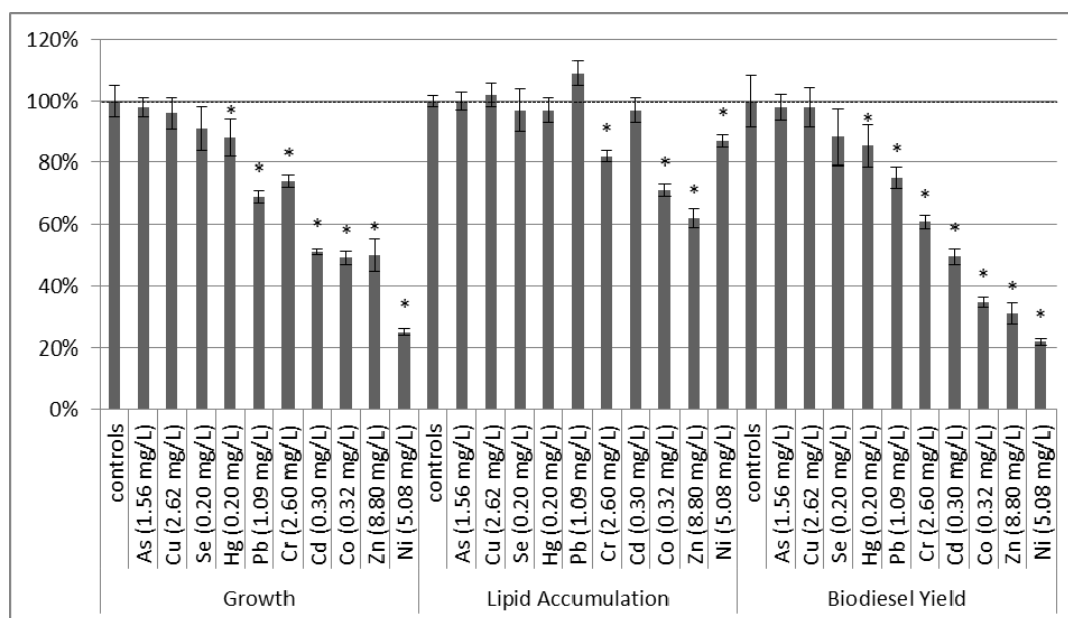


Figure 17. Summary of results for experiments with individual metals at the reference concentrations, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test.

All metals at different concentrations

All metals at 1/20, 1/10, 1/4, and 1/2 the reference concentrations were all tested in the same experiment and therefore have the same control and the results for all of these concentrations will be presented together. Figure 18 shows the growth curve for all of these metals together. The average values with standard deviation are shown as the experiment was conducted in triplicate. The results from Tukey's test are also shown in Figure 18. Table 11 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the growth.

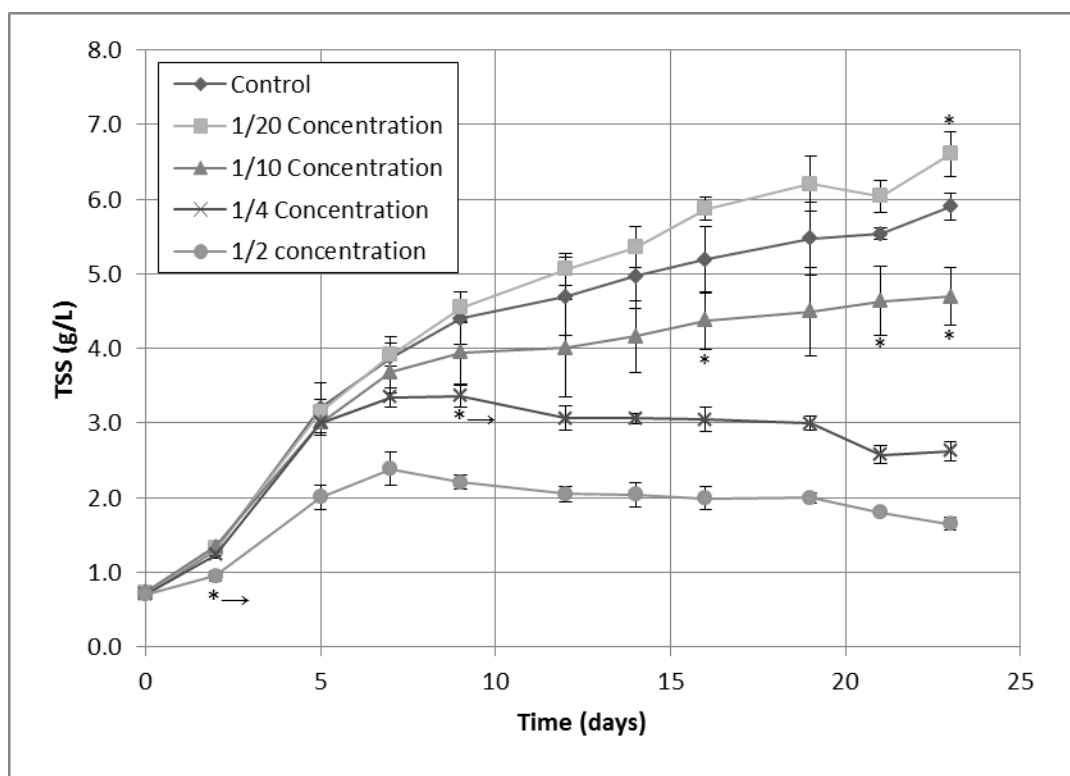


Figure 18. Growth of *Scenedesmus obliquus* with all metals at 1/20, 1/10, 1/4, and 1/2 the reference concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point, the symbol * \rightarrow represents significant difference from control determined by Tukey's test for that point forward.

Table 11. Mean Values \pm 95% Confidence Region for Growth of *Scenedesmus obliquus* with All Metals at 1/20, 1/10, 1/4, and 1/2 Concentration Versus the Control with No Metals

TSS (g/L)	day										
control/ concentration	0	2	5	7	9	12	14	16	19	21	23
control	0.70 ±0.16	1.30 ±0.30	3.20 ±0.84	3.88 ±0.71	4.40 ±0.88	4.69 ±1.29	4.96 ±1.05	5.19 ±1.11	5.47 ±1.22	5.53 ±0.24	5.90 ±0.46
1/20	0.72 ±0.13	1.32 ±0.15	3.14 ±0.44	3.91 ±0.42	4.55 ±0.52	5.06 ±0.55	5.35 ±0.68	5.87 ±0.40	6.20 ±0.92	6.04 ±0.54	6.61 ±0.75
1/10	0.74 ±0.18	1.34 ±0.25	3.00 ±0.43	3.68 ±0.71	3.94 ±1.05	4.01 ±1.62	4.16 ±1.20	4.37 ±0.96	4.49 ±1.49	4.63 ±1.16	4.69 ±0.96
1/4	0.70 ±0.19	1.23 ±0.12	3.00 ±0.18	3.34 ±0.33	3.36 ±0.39	3.06 ±0.43	3.06 ±0.20	3.05 ±0.43	2.99 ±0.27	2.57 ±0.32	2.62 ±0.34
1/2	0.71 ±0.12	0.95 ±0.21	2.00 ±0.42	2.39 ±0.55	2.21 ±0.26	2.05 ±0.28	2.04 ±0.43	1.99 ±0.39	2.00 ±0.20	1.80 ±0.14	1.65 ±0.25

As can be seen from Figure 18, at concentrations of 1/10 the reference concentrations or higher, the metals negatively affected the growth of the algae. However, at 1/20 the reference concentrations the metals slightly enhanced growth. For 1/4 and 1/2 the reference concentrations the metals were obviously very detrimental to the algal growth. The metals at 1/2 the reference concentrations killed the algae, and on day 23 the culture turned brown.

Figure 19 shows the lipid analysis curve for the experiment with all metals at 1/20, 1/10, 1/4, and 1/2 the reference concentrations. The average values with standard deviation are shown as the experiment was conducted in triplicate. The results from Tukey's test are also shown in Figure 19. Table 12 shows the mean values with the 95% confidence region calculated from the uncertainty analysis for the lipid analysis.

As can be seen from Figure 19 different metal concentrations did have a big impact on the lipid content for the algae. At 1/4 and 1/2 the reference concentrations the metals severely decreased the lipid content in the algae after day 16. At 1/10 concentrations there was no significant difference in lipid content compared to the control. At 1/20 concentrations the metals significantly increased lipid content in the algae after day 16. As can be seen from Figure 19, 1/10 concentrations had a huge standard deviation on days 16 and 23. On these days, one of the lipid accumulation values was quite larger than the others. The cause of this difference is not known and there was no similar difference for the growth results. The 1/10 results are included in Figure 19, however, the 1/10 results were not included in Tukey's test, as the large standard deviation interferes with the test's ability to determine a significant difference between the other concentrations and the control.

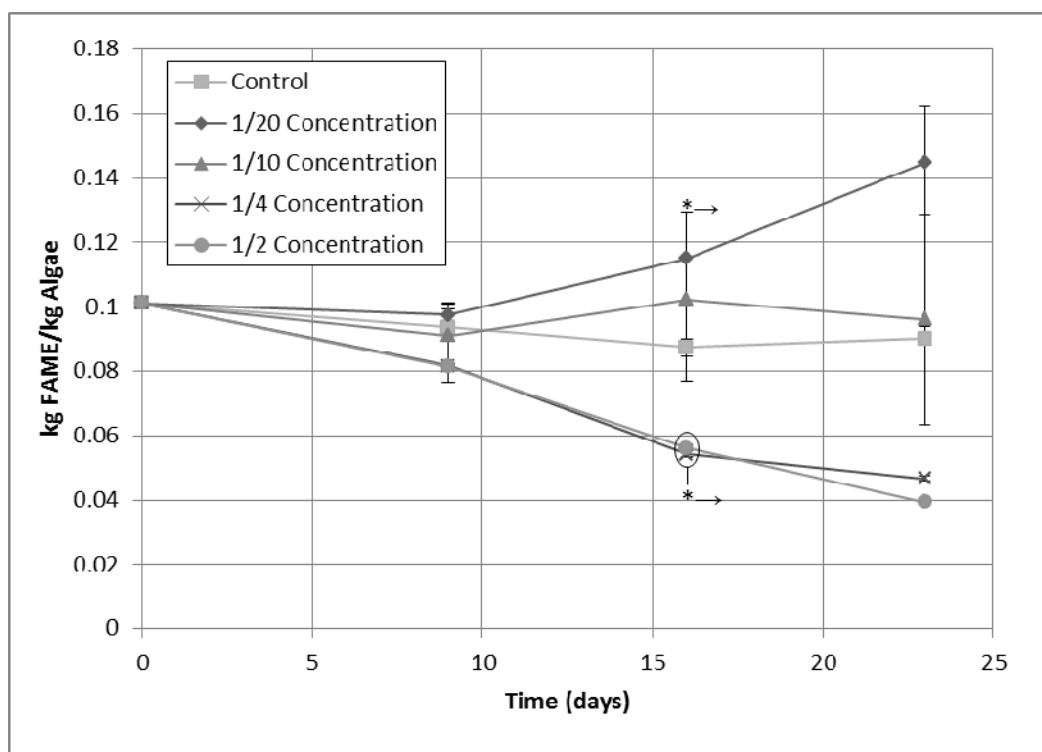


Figure 19. Lipid analysis for *Scenedesmus obliquus* with all metals at 1/20, 1/10, 1/4, and 1/2 the reference concentrations versus the control with no metals, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test at that point, the symbol * \rightarrow represents significant difference from control determined by Tukey's test for that point forward.

Table 12. Mean Values \pm 95% Confidence Region for Lipid Analysis of *Scenedesmus obliquus* with All Metals at 1/20, 1/10, 1/4, and 1/2 Concentration Versus the Control with No Metals

kg FAME/ kg Algae control/ concentration	day			
	0	9	16	23
control	0.1012 ± 0.0074	0.0936 ± 0.0197	0.0874 ± 0.0102	0.0901 ± 0.0119
1/20	0.1012 ± 0.0074	0.0974 ± 0.0089	0.1150 ± 0.0363	0.1448 ± 0.0441
1/10	0.1012 ± 0.0074	0.0912 ± 0.0259	0.1023 ± 0.0631	0.0960 ± 0.0813
1/4	0.1012 ± 0.0074	0.0816 ± 0.0076	0.0542 ± 0.0078	0.467 ± 0.0077
1/2	0.1012 ± 0.0074	0.0815 ± 0.0140	0.0562 ± 0.0078	0.0392 ± 0.0075

Summary of combined metals results

All metals at different concentrations were tested in addition to the experiments with individual metals because it was desired to know what fraction of the reference concentrations the algae could tolerate; another experiment was conducted with all the metals at different fractions of their reference concentrations. The fractions of the reference concentrations that were used were 1/20, 1/10, 1/4, and 1/2.

Figure 20 shows the area under the curves for the experiments with all metals at different concentrations. The control is again set to 100 percent. The standard deviation for the results is shown and the plot is ordered again from highest total lipids produced to lowest total lipids produced. The results from Tukey's test are also shown in Figure 20. Only one experiment was conducted with all metals at different concentrations, and Figure 20 includes the results from this experiment.

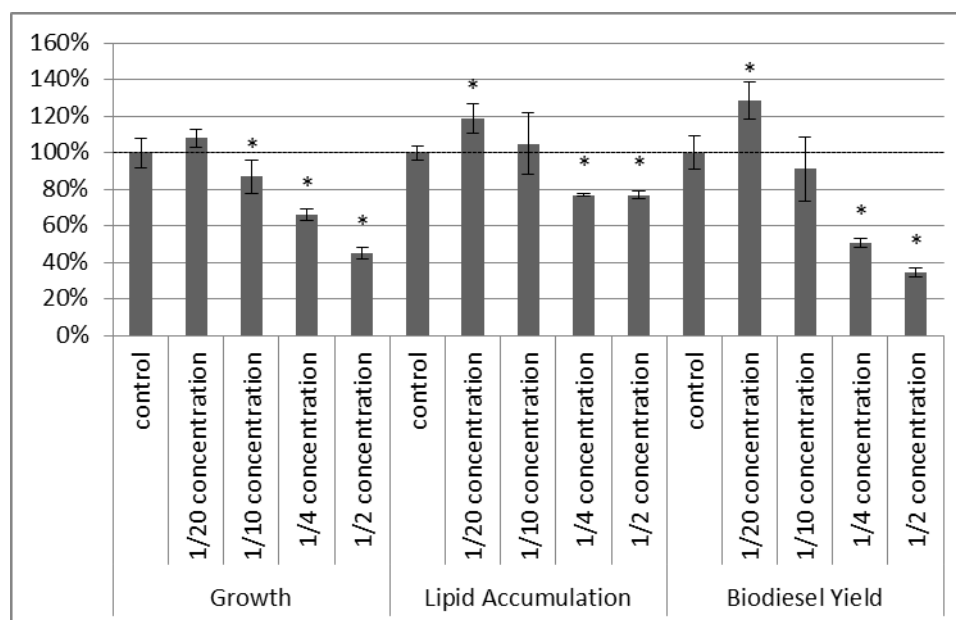


Figure 20. Summary of results for experiments with all metals at different concentrations, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test.

As can be seen from Figure 20 growth and lipid accumulation both declined with an increase in the concentration of metals used. At 1/20 concentration the area under the growth curve and the lipid accumulation curve were both larger than the respective curves for the control without metals. The algae, therefore, produced more biodiesel with small amounts of metals than no metals. The concentrations above 1/20 the reference concentrations all had a negative effect on the algae, and the effects were worse as the concentration rose.

ADDITIONAL DISCUSSION

Concentration of metals

When the experiments began the goal was to understand how each individual metal would impact the growth and lipid accumulation of the algae. The reference concentrations were the first to be tested. As can be seen from the results the initial concentrations were much too high for this strain of algae. Most of the individual metals had a negative effect on the algae and nickel even killed the algae. If the metals were combined, the algae would never be able to handle these concentrations.

The next goal was to find a concentration that would not negatively affect the algae. The original assumption that the metals will build up every time the water is recycled and the water will be recycled 20 times was modified; it was discovered that the metals will probably not build up in this fashion as they are usually absorbed by the algae. All metals were tried at different fractions of the reference concentrations with 1/20 being the lowest fraction. At this concentration the metals appeared to have a positive effect on the algae.

The metal concentrations should be at this level or lower most of the time if algae are grown with flue gas. To show this a comparison was conducted using the literature source used for the calculations and other literature sources. This comparison is shown in Table 13. The comparison includes the following:

1. Estimated concentrations in liquid media using all of the original assumptions except for the last one (this produced 1/20 the reference concentrations, and metals at this level were beneficial to the algae)

2. Estimated concentrations in liquid media using the average mass fractions of different heavy metals instead of the highest mass fractions used for part 1 [41]
3. Estimated concentrations in liquid media using the average mass fractions of different heavy metals from a different source [52]
4. Estimated concentrations in liquid media using the average mass fractions of different heavy metals that are extracted into water using a procedure to estimate the release of heavy metals into natural fluids [52] (this is important as an original assumption was that all of the metals will be leached into the water, but this paper shows that they will not)
5. Estimated concentrations in liquid media based on a proposal from the Environmental Protection Agency (EPA) that was not accepted [53] (calculations for these concentrations will be included in Appendix D as they are quite different from the calculations for the other concentrations).

Table 13. Estimated Concentrations of Metals in Liquid Media (mg/L) Using Various Literature Sources and Assumptions

Metal	(1.)	(2.)	(3.)	(4.)	(5.)
Arsenic	0.08	0.00865	0.01669	0.00409	1.88E-06
Cadmium	0.02	0.00068	<0.00012	0.00010	2.82E-07
Chromium	0.13	0.01795	0.03171	0.00279	2.82E-06
Cobalt	0.02	0.00716	0.00973	0.00037	7.52E-07
Copper	0.13	0.02233	0.02448	0.00280	
Lead	0.05	0.01133	0.01232	0.00046	1.88E-06
Nickel	0.25	0.01547	0.02021	0.00077	3.76E-06
Mercury	0.01	0.00002	0.00002	3.20E-08	
Selenium	0.01	0.00154	<0.00160	0.00121	5.64E-06
Zinc	0.44	0.02951	0.04070	0.00269	

As long as metal concentrations stay below 1/20 of the reference concentrations, heavy metals should not be an issue in terms of producing biodiesel. As can be seen from

the more realistic assumptions in Table 13 concentrations should be quite a bit lower than 1/20 the reference concentrations. By simply using the average mass fractions instead of the highest mass fractions [41] concentrations are decreased by 10 to 500 times as shown under (2.) in Table 13. Using average mass fractions from another source [52] gives similar concentrations to (2.) as shown under (3.) in Table 13. This source also measured how much of the metals will actually be leached into the water and the concentrations are even further decreased as seen under (4.) in Table 13. The concentrations in (4.) are likely closest to what would actually be seen if algae were grown with flue gas. Also, if the EPA had been able to pass the regulations it proposed on control of heavy metal emissions from coal fire plants, concentrations would be even lower.

Using different reactors could change results

Changing the type of reactor used for these experiments will probably change the results. For preliminary experiments, 3L polystyrene reactors were originally being used. The experiments were switched over to the borosilicate tube reactors for several reasons. Heavy metal cations, which include zinc, lead, cobalt, mercury, nickel, copper and cadmium, have a tendency to stick to material surfaces. They stick at least in small part to all materials, but they stick to some materials less than others. Heavy metal cations will stick less to borosilicate than they will to many plastics [54-56]. For the experiments, it was desired that the metals interact with the algae and not stick to the reactor wall, and therefore, the experiments were switched to the borosilicate reactors.

Another reason that the experiments were switched to the borosilicate tube reactors is that more light can penetrate through them than through the 3L polystyrene

reactors causing the algae to grow faster, and allowing the experiments to be completed in less time.

These points are demonstrated in Figure 21, which shows a comparison between growth curves in the polystyrene reactors and the borosilicate tube reactors. The bar graphs represent the area under the growth curves. The results from Tukey's test are also shown in Figure 21.

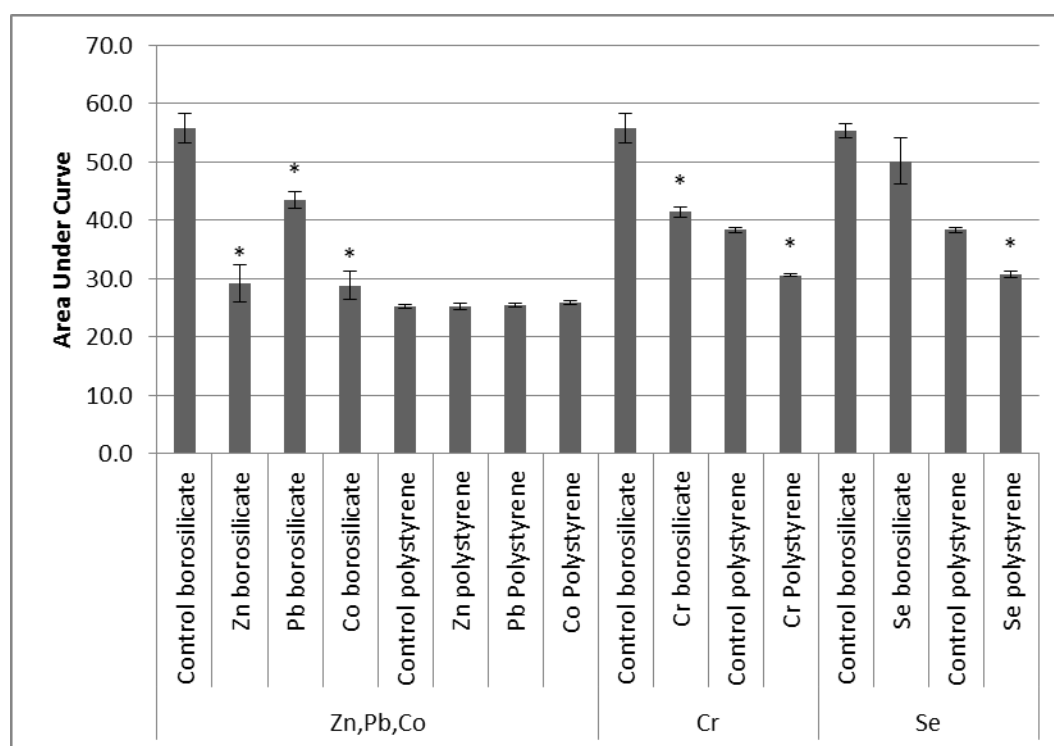


Figure 21. Comparison of growth between polystyrene and borosilicate reactors, mean values \pm SD plotted, the symbol * represents significant difference from control determined by Tukey's test.

As can be seen from Figure 21, there is a significant difference in the growth characteristics for the experiments conducted in the two types of reactors. There are two characteristics of note. The first is that the controls grew much faster in the borosilicate tube reactors. As stated, this is most likely due to better light penetration in the thinner

borosilicate reactors. The second characteristic is that there was no difference in the growth between the control and the algae grown with the cationic metals zinc, lead, and cobalt for the polystyrene reactors, but there was a noticeable difference in the growth between the control and the algae with these metals for the borosilicate reactors. As stated, the most likely reason for this difference is that metals were absorbed on the polystyrene reactor wall more than they were absorbed on the borosilicate reactor wall, and therefore the harmful metals were not interacting with the algae in the polystyrene reactors. As can be seen from Figure 21 the anionic metals chromium and selenium did not show this same characteristic.

Absorption characteristics of cationic metals could be beneficial. If it is desired to limit the exposure of the algae to a certain cationic metal, then an absorbent material such as plastics could be placed in the media to remove some of the metal content.

Using a different strain/media could change results

To see how the results might apply to other strains and media, *Neochloris oleabundans* was grown next to *Scenedesmus obliquus* with the metal selenium. The media used for *Neochloris oleabundans* was SE+ media and will be included in Appendix E, and *Scenedesmus obliquus* used APS media. The comparison is shown in Figure 22. The lipid analysis was also compared for the two strains and is shown in Figure 23.

It should be noted that selenium was selected for this experiment because selenium is the metal that had the most effect on *Scenedesmus obliquus* when the experiments were being conducted in the polystyrene reactors. The comparison between the two strains was the first experiment to be conducted in the borosilicate reactors, and it

was assumed that selenium would again be the most severe of metals, and therefore, differences in the growth and lipid accumulation production between the two strains would be more noticeable. In the borosilicate reactors, however, selenium did not have much effect on growth and lipid accumulation for either strain, as will be shown in the plots in this section. The difference between the two strains would no doubt be more severe had another metal been used for the experiment, however, the differences were enough that it was decided to continue testing the original strain *Scenedesmus obliquus*, and not test *Neochloris oleabundans* any further.

As can be seen from Figure 22 the growth patterns from *Scenedesmus obliquus* and *Neochloris oleabundans* were similar but not exactly the same when grown with selenium. Selenium slightly decreased the growth for *Scenedesmus obliquus*, although only significantly on days six and eight, and did not appear to have any effect on *Neochloris oleabundans*. In addition, growth in *Neochloris oleabundans* started to decrease after day 11. There are several factors that may have caused this. Potassium and nitrogen levels were higher in the media for *Scenedesmus obliquus*. The drop in the growth of *Neochloris oleabundans*, therefore, may have simply been due to lack of nutrients, while *Scenedesmus obliquus* still grew because it still had nutrients. Additionally, it may have simply been due to differences in the growth curves for the two algae. Had the growth continued for more days there could have been a more noticeable difference in the growth curves.

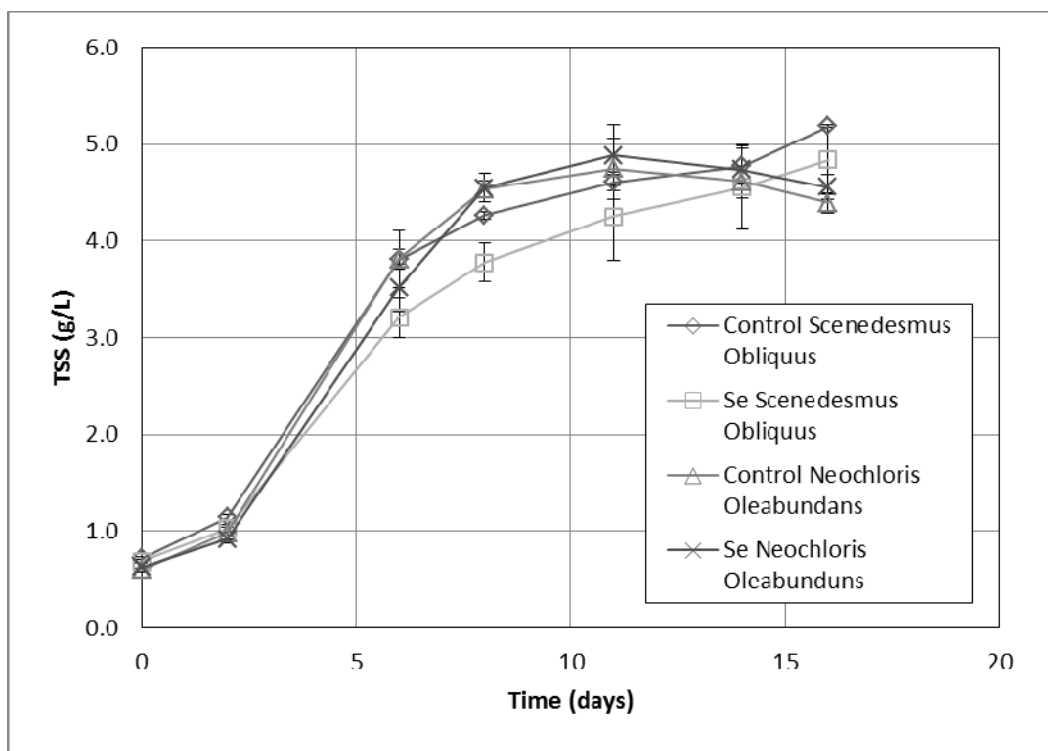


Figure 22. Growth comparison between *Scenedesmus obliquus* and *Neochloris oleabundans* with selenium.

As can be seen from Figure 23 the accumulation of lipids was quite different for the two strains. *Neochloris oleabundans* had much higher lipid content than *Scenedesmus obliquus*. However the effect of selenium on lipid was the same for both strains; there was no change from the control.

Overall, the results from the two strains were similar, but there were differences. Using other strains and media may produce similar results, but there will be differences, and the results from this study should be used only as a reference.

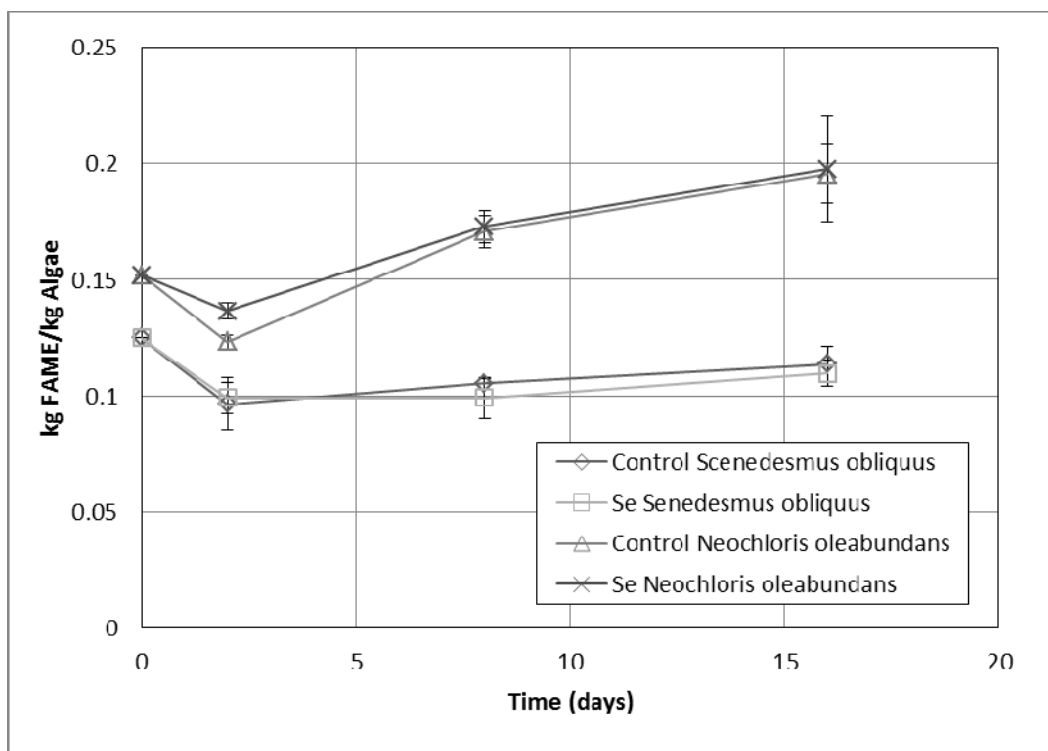


Figure 23. Lipid analysis comparison between *Scenedesmus obliquus* and *Neochloris oleabundans* with selenium.

Presence of chelators could change results

APS media contains an fe-EDTA portion that was not included for the experiments with the algae because the EDTA binds with the metals and renders them non-bio-available to the algae. In addition, the algae were centrifuged and washed with fresh media before starting a new experiment to remove any chelators that the algae naturally produced. These steps were done to increase the metals' interaction with the algae; however, this is not practical outside of a laboratory setting.

Because chelators will bind with metals, the algae might be able to handle higher concentrations of metals if more chelators are present. This could be accomplished by adding chelators to the media and not interfering with chelators that the algae naturally produce.

Differences in pH could change results

Heavy metals will stay in ionic form at very low pH, but as the pH is increased the metals form into precipitates that will not be bio-available to the algae. In addition, one study found that several cationic metals will not even leach into water from flue gas fly ash if the pH of the water is between 8 and 11; this study used a procedure that estimated the release of heavy metals into natural fluids [52]. For these experiments the pH was approximately 7, but using a pH above 8 could be advantageous if the algae used can tolerate a higher pH.

CONCLUSIONS

Metals in flue gas should not affect production of biodiesel for the algae strain used in this experiment. The original reference concentrations are extreme and if flue gas is used to grow algae they will most likely not be exposed to concentrations that high. At 1/20 of the reference concentrations, the metals were beneficial to algae, and improved both growth and lipid accumulation. Based on a review of other sources to estimate the concentration of metals the algae will be exposed to, it is concluded that the concentrations will most likely be under 1/20 of the reference concentrations. These concentrations are shown in Table 14.

Table 14. Concentration of Each Metal That Was Beneficial to the Algae

Component	Conc. In Liquid Media (mg/L)
Arsenic	0.08
Cadmium	0.02
Chromium	0.13
Cobalt	0.02
Copper	0.13
Lead	0.05
Nickel	0.25
Mercury	0.01
Selenium	0.01
Zinc	0.44

If metal concentrations were to rise above the values shown in Table 14, however, the metals could start negatively affecting the algae. All metals together at 1/10, 1/5, and 1/2 of the reference concentrations had a negative effect on the growth of the algae, and to a lesser extent the accumulation of lipids. The negative effect was greater as the concentrations increased.

The metals were also tested individually at the reference concentration; even individually, at these concentrations most of the metals had a negative effect on the algae as shown in Table 15. It is concluded that all of the metals tested, whether individual or combined, are toxic to the algae when the metals are above a certain concentration. At the reference concentrations, most of the metals tested are above that limit. Below 1/20 the reference concentration all of the metals tested do not negatively affect the algae.

Table 15. Individual Metals at 1/20 the Reference Concentrations and Their Effect on the Algae

Metal	Concentration	Effect on Growth	Effect on Lipid Accumulation
Arsenic	1.56 mg/L	no significant effect	no significant effect
Cadmium	0.30 mg/L	drastically decreased	no significant effect
Chromium	2.60 mg/L	significantly decreased	slightly decreased
Cobalt	0.32 mg/L	drastically decreased	significantly decreased
Copper	2.62 mg/L	no significant effect	no significant effect
Lead	1.09 mg/L	significantly decreased	slightly increased
Nickel	5.08 mg/L	severely decreased/ killed algae	slightly decreased
Mercury	0.20 mg/L	slightly decreased	no significant effect
Selenium	0.20 mg/L	slightly decreased	no significant effect
Zinc	8.80 mg/L	drastically decreased	significantly decreased

Another conclusion of this study is there are many different factors that could be implemented to control the concentrations of metals to which the algae are exposed. These factors include exposing the algae to highly absorptive materials such as plastics, using chelators to bind the metals, or increasing the pH of the reactor. In summary, if concentrations should rise above the desired levels there are methods that can be used to decrease concentrations to more acceptable levels.

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APPENDICES

APPENDIX A

APS Freshwater Medium preparation

Date:
Name:

1. Fe-EDTA solution

Chemical Component	Needed per 1L media	To make 1L of 1000X stock solution	Add (g or mL)	Actually added (g)	Final Prep
Na ₂ EDTA*2H ₂ O	0.012000	1000 X	12		Use 1ml of this stock per
FeSO ₄ *7H ₂ O	0.004500	1000 X	4.5		1ml of media that you
10M NaOH	0.005000	1000 X	5		make

Fill a clean 1L beaker with 0.9 L deionized or distilled water, mix components and very slowly titrate with 10M NaOH until all the precipitate has dissolved. Bring final volume to 4 liters with deionized or distilled water. Transfer the solution to a labeled clean 5L media bottle and autoclave

2. Micronutrient solution

Chemical Component	Needed per 1L media	To make 1L of 1000X stock solution	Add (g or mL)	Actually added (g)	Final Prep
ZnSO ₄ *7H ₂ O	0.000086	1000 X	0.086		Use 1ml of this stock per 1ml of media that you make
MnCl ₂ *4H ₂ O	0.000597	1000 X	0.597		
Na ₂ MoO ₄ *2H ₂ O	0.000058	1000 X	0.058		
CuCl ₂ *2H ₂ O	0.000041	1000 X	0.041		
CoCl ₂ *6H ₂ O	0.000029	1000 X	0.029		
MgSO ₄ *7H ₂ O	0.049113	1000 X	49.113		
MgCl ₂ *6H ₂ O	0.021454	1000 X	21.454		
CaCl ₂ *2H ₂ O	0.025050	1000 X	25.050		
H ₃ BO ₃	0.011400	1000 X	11.400		

Fill a clean 2L beaker about halfway with deionized or distilled water, weigh the listed components. Mix components and bring to final volume of 2 L with deionized or distilled water. Transfer the solution to a labeled clean 2L media bottle and autoclave.

3. Preparing Macronutrient portion

Chemical Component	Needed per 1L media	To make 1L of 1000X stock solution	Add (g or mL)	Actually added (g)	Final Vessel
NaNO ₃	1.000000	1000 X	1		4kg bag of nitrate
FeSO ₄ *7H ₂ O	0.200000	1000 X	0.2		800g bag of phosph.

Fill a clean 1L bottle with 900mL water, add these components, then add 1ml of each stock solution, and bring up to 1L.

APPENDIX B

Percent carbon in coal (Hong et al.).

$$P_{cc} := 0.6$$

Total ash content of coal and Fly ash content of total ash (Coles et al., Babcock and Wilcox).

$$P_{Tash} := 0.22 \quad P_{Fash} := 0.8$$

Density of CO2 at atmospheric pressure

$$D_{CO2} := 1.977 \frac{\text{gm}}{\text{L}}$$

Percent carbon in CO2 by mass

$$P_c := 0.2729$$

mass of fly ash per liter of flue gas CO2 (assuming all carbon in coal is converted to CO2).

$$MD_{fg} := \frac{P_{Tash} \cdot P_{Fash}}{P_{cc}} \cdot D_{CO2} \cdot P_c \quad MD_{fg} = 0.158 \frac{\text{gm}}{\text{L}}$$

Percentage of Fly ash entering reactor per liter CO2 (Strand et al.).

$$P_{FashR} := 0.01 \quad MD_{FR} := MD_{fg} \cdot P_{FashR} \quad MD_{FR} = 1.583 \times 10^{-3} \frac{\text{gm}}{\text{L}}$$

Flow rate for CO2 entering into our 8 reactors (approximate observance).

$$F_C := 150 \frac{\text{mL}}{\text{min}}$$

Volume in 8 reactors

$$V_R := 3\text{L} \cdot 8 \quad V_R = 24\text{L}$$

Actual Flow Rate seen in our experiments (in vvm therefore there are no volume units).

$$F_R := \frac{F_C}{V_R} \quad F_R = 6.25 \times 10^{-3} \frac{1}{\text{min}}$$

Concentration in reactor assuming a 14 day growing cycle

$$MD_R := F_R \cdot MD_{FR} \cdot 14\text{day} \quad MD_R = 0.199 \frac{\text{gm}}{\text{L}}$$

Highest possible Mass fraction of different metal components (Committee on Mine Placement of Coal Combustions Wastes 2006).

$$\begin{aligned}
 MF_{\text{arsenic}} &:= 391.0 \frac{\text{mg}}{\text{kg}} & MF_{\text{lead}} &:= 273 \frac{\text{mg}}{\text{kg}} \\
 MF_{\text{cadmium}} &:= 76.0 \frac{\text{mg}}{\text{kg}} & MF_{\text{nickel}} &:= 1270 \frac{\text{mg}}{\text{kg}} \\
 MF_{\text{chromium}} &:= 651.0 \frac{\text{mg}}{\text{kg}} & MF_{\text{mercury}} &:= 49.5 \frac{\text{mg}}{\text{kg}} \\
 MF_{\text{cobalt}} &:= 79 \frac{\text{mg}}{\text{kg}} & MF_{\text{selenium}} &:= 49.5 \frac{\text{mg}}{\text{kg}} \\
 MF_{\text{copper}} &:= 655 \frac{\text{mg}}{\text{kg}} & MF_{\text{zinc}} &:= 2200 \frac{\text{mg}}{\text{kg}}
 \end{aligned}$$

Concentration after one cycle and twenty cycles

$$\begin{aligned}
 MD_{\text{arsenic}} &:= MF_{\text{arsenic}} \cdot MD_R & MD_{\text{arsenic}} &= 0.078 \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{arsenic}} \cdot 20 &= 1.559 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{cadmium}} &:= MF_{\text{cadmium}} \cdot MD_R & MD_{\text{cadmium}} &= 0.015 \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{cadmium}} \cdot 20 &= 0.303 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{chromium}} &:= MF_{\text{chromium}} \cdot MD_R & MD_{\text{chromium}} &= 0.13 \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{chromium}} \cdot 20 &= 2.596 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{cobalt}} &:= MF_{\text{cobalt}} \cdot MD_R & MD_{\text{cobalt}} &= 0.016 \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{cobalt}} \cdot 20 &= 0.315 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{copper}} &:= MF_{\text{copper}} \cdot MD_R & MD_{\text{copper}} &= 0.131 \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{copper}} \cdot 20 &= 2.612 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{lead}} &:= MF_{\text{lead}} \cdot MD_R & MD_{\text{lead}} &= 0.054 \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{lead}} \cdot 20 &= 1.089 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{nickel}} &:= MF_{\text{nickel}} \cdot MD_R & MD_{\text{nickel}} &= 0.253 \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{nickel}} \cdot 20 &= 5.065 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{mercury}} &:= MF_{\text{mercury}} \cdot MD_R & MD_{\text{mercury}} &= 9.871 \times 10^{-3} \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{mercury}} \cdot 20 &= 0.197 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{selenium}} &:= MF_{\text{selenium}} \cdot MD_R & MD_{\text{selenium}} &= 9.871 \times 10^{-3} \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{selenium}} \cdot 20 &= 0.197 \cdot \frac{\text{mg}}{\text{L}} \\
 MD_{\text{zinc}} &:= MF_{\text{zinc}} \cdot MD_R & MD_{\text{zinc}} &= 0.439 \cdot \frac{\text{mg}}{\text{L}} & MD_{\text{zinc}} \cdot 20 &= 8.774 \cdot \frac{\text{mg}}{\text{L}}
 \end{aligned}$$

APPENDIX C

Daniel Nelson, Utah State University

Updated 17 June 2009

Acid-Catalyzed In-situ transesterification Reaction Using Waterbath (or similar heating method)

1. Weight out desired biomass amount into crimp top GC vials. (Use lyophilized biomass unless you wish to address effects of water on the system).
2. Into each vial, add 0.5 of acidified methanol (5% H₂SO₄, or other concentrations of your choosing.)
3. Vial is sealed and placed in heating source (90°C) for 30-180 minutes.
 - a. Optimal time for most organisms is 60 minutes.
 - b. This optimal time is based on 5% acid, and up to 60mg biomass/ml methanol, at 90°C.
4. After 30 minutes, the vials can be removed and shaken every 15 to keep solution suspended.
 - a. This step is not necessary unless large biomass/solvent ratios are being used.
5. After desired heating time, the vial is removed and centrifuged to prevent biomass from being pipetted into the next stage.
6. In a separate 5ml serum bottle, 4ml hexane is added and the 0.5ml of the acidified methanol solution containing the FAMES is also added.
 - a. Be sure to remove the entire amount of methanol from the GC vial as this contains all of the FAMES.
7. Using an additional 1ml of Hexane, the GC vial containing the residual biomass is washed. Remove the 1ml of Hexane and add it to the 5ml serum bottle, bringing the entire volume to 5.5ml (~5ml Hexane, 0.5ml Methanol).
 - a. The washing step is done to extract and collect all remaining amounts of fatty acids that might have been deposited on the biomass when the methanol was removed (can be as much as 5%).
8. The serum bottle is sealed with a Teflon lined cap and heated (usually water bath) at 90°C for 15 min.
 - a. This step is proven to extract >95% of the total fatty acids present in the methanol.
 - b. If done less than 10 minutes up to 20% FAMES may not be recovered, longer than 20-30 minutes and product degradation can be seen.
9. The 5ml serum bottle is removed from the heat and cooled to room temperature to allow the phases to separate (hexane-FAMES, methanol).
10. The upper phase, hexane, containing the FAME's is then pipetted to a separate auto-sampler GC vial for analysis.
 - a. Depending on the amount of lipids present in the system, different dilutions are needed. Generally, the desired concentration is 0.1mgFAME/ml solvent. If the organism is 50% FAME (w/w), at least a 1:10 dilution with hexane is necessary.
11. To each sample, an internal standard is added. The type of internal standard is up to the researcher.
12. Standard calibration curves of each target compound needs to be determined if quantification is desired.

APPENDIX D

Percent carbon in coal (Hong et al.).

$$P_{cc} := 0.6$$

Density of CO2 at atmospheric pressure

$$D_{CO2} := 1.977 \frac{\text{gm}}{\text{L}}$$

Percent carbon in CO2 by mass

$$P_c := 0.2729$$

Volume CO2 per mass coal

$$V_{CO2} := \frac{P_{cc}}{D_{CO2} P_c} \quad V_{CO2} = 1.112 \frac{\text{L}}{\text{gm}}$$

BTU/lb coal (EPA 2011) and Volume CO2 per btu coal

$$\text{Energy}_{\text{coal}} := 8300 \frac{\text{BTU}}{\text{lb}}$$

$$V_{CO2} := \frac{V_{CO2}}{\text{Energy}_{\text{coal}}} \quad V_{CO2} = 0.061 \frac{\text{L}}{\text{BTU}}$$

Flow rate for CO2 entering into our 8 reactors (approximate observance).

$$F_C := 150 \frac{\text{mL}}{\text{min}}$$

Volume in 8 reactors

$$V_R := 3\text{L} \cdot 8 \quad V_R = 24\text{L}$$

Actual Flow Rate seen in our experiments (in vvm therefore there are no volume units).

$$F_R := \frac{F_C}{V_R} \quad F_R = 6.25 \times 10^{-3} \frac{1}{\text{min}}$$

BTUs created per liter of reactor to produce CO2 for alga for a 14 day growing cycle

$$MD_R := \frac{F_R}{V_{CO2}} \cdot 14\text{day} \quad MD_R = 2.073 \times 10^3 \frac{\text{BTU}}{\text{L}}$$

Proposed emissions limits for coal (EPA 2011)

$$\begin{aligned}
 \text{Em}_{\text{As}} &:= \frac{2.0\text{lb}}{10^{12}\text{BTU}} & \text{Em}_{\text{Cd}} &:= \frac{0.3\text{lb}}{10^{12}\text{BTU}} \\
 \text{Em}_{\text{Cr}} &:= \frac{3.0\text{lb}}{10^{12}\text{BTU}} & \text{Em}_{\text{Co}} &:= \frac{0.8\text{lb}}{10^{12}\text{BTU}} \\
 \text{Em}_{\text{Pb}} &:= \frac{2.0\text{lb}}{10^{12}\text{BTU}} & \text{Em}_{\text{Ni}} &:= \frac{4.0\text{lb}}{10^{12}\text{BTU}} \\
 \text{Em}_{\text{Se}} &:= \frac{6\text{lb}}{10^{12}\text{BTU}}
 \end{aligned}$$

Final Concentration in liquid media

$$\begin{aligned}
 \text{MD}_{\text{As}} &:= \text{Em}_{\text{As}} \cdot \text{MD}_{\text{R}} & \text{MD}_{\text{As}} &= 1.881 \times 10^{-6} \frac{\text{gm}}{\text{L}} \\
 \text{MD}_{\text{Cd}} &:= \text{Em}_{\text{Cd}} \cdot \text{MD}_{\text{R}} & \text{MD}_{\text{Cd}} &= 2.821 \times 10^{-7} \frac{\text{gm}}{\text{L}} \\
 \text{MD}_{\text{Cr}} &:= \text{Em}_{\text{Cr}} \cdot \text{MD}_{\text{R}} & \text{MD}_{\text{Cr}} &= 2.821 \times 10^{-6} \frac{\text{gm}}{\text{L}} \\
 \text{MD}_{\text{Co}} &:= \text{Em}_{\text{Co}} \cdot \text{MD}_{\text{R}} & \text{MD}_{\text{Co}} &= 7.523 \times 10^{-7} \frac{\text{gm}}{\text{L}} \\
 \text{MD}_{\text{Pb}} &:= \text{Em}_{\text{Pb}} \cdot \text{MD}_{\text{R}} & \text{MD}_{\text{Pb}} &= 1.881 \times 10^{-6} \frac{\text{gm}}{\text{L}} \\
 \text{MD}_{\text{Ni}} &:= \text{Em}_{\text{Ni}} \cdot \text{MD}_{\text{R}} & \text{MD}_{\text{Ni}} &= 3.762 \times 10^{-6} \frac{\text{gm}}{\text{L}} \\
 \text{MD}_{\text{Se}} &:= \text{Em}_{\text{Se}} \cdot \text{MD}_{\text{R}} & \text{MD}_{\text{Se}} &= 5.642 \times 10^{-6} \frac{\text{gm}}{\text{L}}
 \end{aligned}$$

APPENDIX E

SE+ Media:			Batch Volume [L] =		3.5
Constituent	mass (mg) per liter	mass (gm) per liter	Mass to add [mg]	Mass to add [gm]	Checklist
DI Water [mL]	999	999	3496.5	3496.5	
Potassium Phosphate Dibasic	150	0.15	525	0.525	
Magnesium Sulfate Heptahydrate	150	0.15	525	0.525	
Calcium Chloride Dihydrate	50	0.05	175	0.175	
Potassium Phosphate Monobasic	350	0.35	1225	1.225	
Sodium Chloride	50	0.05	175	0.175	
Sodium Nitrate	850	0.85	2975	2.975	
Ammonium Ferric Citrate	15	0.015	52.5	0.0525	
1000X Micronutrient Solution [mL]	1	1	3.5	3.5	
Adjust pH to 7.5					

Agar for streak plates: 12 gm / Liter

SE+ Micronutrients:			Batch Volume [L] =		0.2
Constituent	mass (mg) per liter	mass (gm) per liter	Mass to add [mg]	1000 X [mg]	Checklist
DI Water [mL]	1000	1000	200	200	
H3BO3	2.86	0.00286	0.572	572	
MnCl2·4H2O	1.81	0.00181	0.362	362	
ZnSO4·7H2O	0.22	0.00022	0.044	44	
CuSO4·5H2O	0.079	0.000079	0.0158	15.8	
(NH4)6Mo7O24·4H2O	0.039	0.000039	0.0078	7.8	